EMPLOYING HOST-SELECTED ENVIRONMENTAL MICROBES FOR HEALTH BENEFITS TO PLANTS UNDER WATER DEFICIT

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the requirements for the degree of

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IN

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SUBMITTED BY

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Sincerely,

Shilpi Sharma 2nd June 2025
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DECLARATION BY THE STUDENT

I hereby declare that the work being presented in this dissertation entitled **"Employing host-selected environmental microbes for health benefits to plants under water deficit"** in partial fulfilment of the requirements for the award of the degree of **Master of Science in Biotechnology**, Sister Nivedita University, is an authentic record of my own work carried out during the period from **3rd February 2025** to **3rd June 2025** under the supervision of **Dr. Shilpi Sharma**, Batch of 1980 Chair Professor, Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology Delhi, New Delhi.

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Abbreviations

16S rRNA	16S ribosomal ribonucleic acid
ACC	1-aminocyclopropane-1-carboxylate
DF	Dworkin and Foster
EPS	Exopolysaccharide
IAA	indole-3-acetic acid
LBA	Luria-Bertani agar
NA	nutrient Agar
NBRIP	National Botanical Research Institute’s phosphate
PEG	polyethylene glycol
PGPR	plant growth-promoting rhizobacteria
PGC	plant growth cycle
SynCom	synthetic microbial community
TRE	tomato root exudate
TSB	tryptic soy broth

Abstract

Drought stress remains a significant obstacle in achieving sustainable agriculture, which seeks to maintain productivity under environmental constraints. Traditional chemical strategies often pose environmental risks or have limited long-term effectiveness, highlighting the need for biological approaches to enhance crop resilience. This work investigates the potential of plant growth-promoting rhizobacteria (PGPR) to improve drought tolerance in plants. Tomato (*Solanum lycopersicum*) was used as a model system in this study.

An experiment was conducted to simulate repeated drought exposure across successive plant growth cycles (PGCs). Analysis of biochemical stress markers in tomato plants indicated enhanced physiological adaptation by the third cycle. Rhizospheric soil collected after the first PGC served as the source for PGPR isolation. Ten bacterial isolates were cultured on standard nutrient media and a tomato root exudate (TRE)-mimicking medium, and screened for drought tolerance under polyethylene glycol (PEG)-induced osmotic stress conditions.

Key plant growth-promoting traits—such as exopolysaccharide production, indole-3-acetic acid synthesis, ACC deaminase activity, phosphate solubilization, and siderophore production—were evaluated through a combination of qualitative and quantitative assays. Isolate compatibility was assessed via cross-streaking to support future synthetic community (SynCom) development.

This study demonstrates a structured approach to selecting and characterizing drought-resilient PGPR, providing a foundation for the future development of SynComs and sustainable bioformulations aimed at mitigating drought stress in agriculture.

Keywords: Drought stress, multiple passaging, plant growth-promoting rhizobacteria

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1. Introduction

The increasing frequency and severity of drought events due to global climate change pose a significant threat to agricultural productivity and food security worldwide (Naumann *et al*., 2021). Drought stress not only suppresses plant growth and crop yields but also disrupts soil health and microbial communities, both of which are critical for plant-microbe interactions and overall ecosystem functioning (Naylor and Coleman-Derr, 2018). According to FAO (2021), the increasing frequency and intensity of droughts, driven by climate change, pose a major threat to global crop production by diminishing the availability and productivity of land and water resources. Therefore, developing effective and sustainable strategies to enhance drought tolerance in crops is crucial for ensuring agricultural stability under increasingly erratic climatic conditions.

Traditional methods to combat drought—such as genetic modification, conventional breeding, and chemical treatments—have shown some success but often fall short in terms of scalability, cost-effectiveness, and ecological sustainability. In response, researchers are increasingly exploring microbe-based interventions, particularly those involving plant-associated beneficial microbes, as promising alternatives for improving plant resilience under drought conditions. Microbe-based approaches, which typically rely on the inoculation of single beneficial strains (e.g., *Azospirillum*, *Bacillus*, or *Pseudomonas*), have demonstrated potential for improving drought tolerance by enhancing nutrient uptake, hormone production, and stress mitigation (Ngumbi and Kloepper, 2016). However, such approaches often struggle in field environments due to inconsistent colonization and competition with native soil microbiota.

To overcome these challenges, microbiome-based agriculture has emerged as a more holistic alternative, focusing not just on single strains but on modulating or engineering the entire rhizosphere microbial community for enhanced plant resilience. While individual strains can confer drought tolerance through mechanisms like phytohormone production (e.g., indole-3-acetic acid (IAA), abscisic acid (ABA), nutrient solubilization, osmolyte synthesis, and activation of antioxidant defences, communities offer synergistic and more stable effects by enabling functional redundancy, microbial cooperation, and ecological balance. The rhizosphere microbiome—comprising dynamic populations of bacteria, fungi, and archaea—thus plays a pivotal role in modulating plant responses to abiotic stresses. (Bittencourt *et al*., 2023).

To address the limitations of microbial inoculants under field conditions, researchers have proposed approaches such as rhizosphere acclimatization and multi-cycle drought passaging. Although they may appear similar, their underlying principles differ significantly. Rhizosphere acclimatization involves the passive, natural evolution of microbial communities in response to prolonged exposure to drought conditions in the field, without human intervention. Over time, this process enables the microbiome to adapt organically to environmental stress. In contrast, multi-cycle drought passaging is an active, controlled strategy where plants and their root-associated microbiota are deliberately subjected to repeated drought stress across several plant generations in experimental setups. This selective pressure helps enrich for specific microbial strains that enhance drought tolerance. (Zolla *et al*., 2013).

Such multi-passaging approaches have been successfully demonstrated in crops like maize (Naylor *et al*., 2017) and rice (Santos-Medellín *et al*., 2021), where drought-adapted microbiomes were enriched and associated with improved plant performance. However, their application in tomato remains largely underexplored—despite its dual relevance as both a widely used model organism in plant research and a water-intensive crop often cultivated in greenhouses, making it particularly susceptible to drought-related productivity losses. One such regionally adapted cultivar is Pusa Sadabahar, which has been widely used in Indian agriculture. This variety was selected for the present study due to its consistent fruiting performance throughout the year and its economic relevance to smallholder farmers.The present study aims to address this gap by isolating and characterizing drought-adapted bacterial strains from the rhizosphere of Pusa Sadabahar plants subjected to successive drought cycles. These isolates were evaluated for key plant growth-promoting traits, including IAA production, 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, siderophore production, exopolysaccharide (EPS) production, and phosphate solubilization. Concurrently, stress markers such as proline accumulation, and malondialdehyde (MDA) levels were assessed to track plant responses across cycles.

By integrating microbial screening with physiological monitoring of stress markers such as proline accumulation and malondialdehyde (MDA) levels, this research aims to lay the groundwork for a microbe-assisted drought mitigation strategy in tomato, which could eventually evolve into a more comprehensive microbiome engineering approach. While the current focus is on a regionally adapted cultivar, the insights gained have the potential to inform scalable and sustainable strategies for climate-resilient agriculture both within India and globally.

2. Review of Literature

2.1. Arable land under drought

Drought significantly impacts arable land, affecting agricultural productivity and livelihoods worldwide. A study by Ge *et al*. (2012) found that water stress during drought periods slows down crop root growth, delays maturation, and reduces agricultural productivity. This is especially critical because the sensitivity of crops to water stress differs depending on their stage of development, making certain growth phases more vulnerable to drought.

In India, approximately two-thirds of the country is prone to drought, making it highly vulnerable to such events. The "Drought Atlas of India, 1901–2020" provides a comprehensive analysis of drought patterns over more than a century, highlighting the persistent challenge drought poses to Indian agriculture (Chuphal *et al*., 2024). The Indo-Gangetic plain, a crucial agricultural region in India, has experienced recurrent drought events over the past few decades, particularly affecting states like Punjab, Haryana, and Uttar Pradesh. These droughts have led to substantial reductions in crop yields, thereby impacting both the agricultural sector and the broader economy. Developing countries, especially those in arid and semi-arid regions, are highly susceptible to drought-induced food insecurity and economic disruption. Research indicates that these regions face increasing vulnerability due to the severe impacts of drought on crop yields and subsistence farming (Naumann *et al*., 2021). In the Indian context, the Indo-Gangetic plain stands out as a critical area suffering from recurrent droughts, resulting in decreased agricultural productivity and heightened vulnerability of rural communities. Comprehensive mitigation strategies, including sustainable water management, drought-resilient crops, and robust early warning systems, are essential to address these challenges.

2.2. Effects of drought stress on plants

Drought stress significantly impacts plant physiology and biochemistry, manifesting through alterations in pigment composition, oxidative stress, cell membrane stability, and the accumulation of proline and other stress markers. One of the primary responses to drought is the degradation of chlorophyll pigments, particularly chlorophyll a and b, which reduces photosynthetic efficiency and impairs plant growth and yield. This degradation acts a protective mechanism to minimize light absorption and avoid photo-oxidative damage under limited water availability (Ashrafand Harris 2013). Concurrently, drought induces oxidative stress by generating an overabundance of reactive oxygen species (ROS), such as superoxide anions, hydrogen peroxide, and hydroxyl radicals, which disrupt cellular homeostasis by

damaging lipids, proteins, and nucleic acids. The imbalance between ROS production and antioxidant defence systems intensifies lipid peroxidation, particularly in cell membranes, leading to increased membrane permeability, ion leakage, and metabolic dysfunction (Gilland Tuteja2010). Proline, a widely recognized abiotic stress marker, accumulates in response to drought and plays a multifaceted role adaptation. It functions as an osmoprotectant by maintaining cellular osmotic balance, stabilizing membranes and proteins, scavenging ROS, and acting as a signalling molecule to regulate stress-responsive gene expression (Szabados and Savouré 2010). These physiological and biochemical responses collectively reflect the complexity of drought tolerance mechanisms in plants, emphasizing the importance of understanding these pathways to develop drought-resilient crop varieties.

2.3. Microbial diversity of drought-affected rhizosphere

Drought stress significantly alters microbial diversity and community structure in the rhizosphere, the narrow soil zone influenced by plant roots. Under normal moisture conditions, the rhizosphere harbors a diverse assemblage of bacteria, fungi, and archaea that drive nutrient cycling, organic matter decomposition, and plant growth (Philippot *et al*., 2013). Taxa such as *Pseudomonas*, *Bacillus*, and *Rhizobium* flourish in this environment, maintaining a functionally active soil milieu. However, prolonged water deficit disrupts this equilibrium, reducing overall richness and shifting communities toward drought-tolerant groups. For instance, in greenhouse pot trials with cassava, a 14-day withholding of irrigation caused a marked decline in bacterial α-diversity in rhizosphere soil compared to well-watered controls (Huang *et al*., 2024). Naylor *et al*. (2017) similarly demonstrated that drought and host genotype jointly reshape grass root bacterial communities. Under desiccation, spore-forming Actinobacteria and certain fungi dominate because they produce durable cell walls, accumulate compatible solutes like trehalose, and form spores that withstand low water potentials. This shift can compromise key functions—such as nitrogen fixation and phosphorus solubilization—performed by more sensitive microbes. Nevertheless, some rhizosphere inhabitants counter drought by secreting large amounts of exopolysaccharide (EPS) that form moisture-retentive biofilms or by entering dormant states (e.g., spores), strategies that enhance their survival and persistence under water limitation. These adaptive traits highlight their promise as microbial inoculants to reinforce plant resilience in drought-stressed soils.

2.4. Conventional approaches to combat drought stress in crops

Conventional strategies to combat drought stress in crops encompass soil management practices, bio-inoculation, and genetic engineering. Soil management techniques such as mulching, tillage and the use of organic amendments are commonly employed to conserve soil moisture, enhance water retention, and improve soil structure. These practices reduce evaporation and improve the infiltration of water into the soil, thus helping plants cope with drought. Additionally, bio-inoculation with beneficial microbes has emerged as a promising strategy to enhance plant drought tolerance. Single-strain inoculation, including drought-resistant bacteria like *Azospirillum, Pseudomonas,* and *Streptomyces*, has been shown to improve plant growth under water-limited conditions by enhancing nutrient availability and modulating plant stress responses(Cohen et al., 2015; Nishu et al., 2022) .

Furthermore, genetic engineering approaches, particularly gene knockdown and knockout techniques, have been utilized to improve drought tolerance by modifying pathways involved in stress perception, signal transduction, osmotic adjustment, and root system development. For instance, the overexpression of the *DREB1A* transcription factor in *Arabidopsis* has been shown to enhance drought tolerance by activating a cascade of downstream genes involved in dehydration-responsive pathways, thereby improving water retention and stress adaptation mechanisms (Kasuga et al., 1999). These conventional and biotechnological approaches, when combined, present a holistic strategy for mitigating drought-induced stress in agriculture.

2.5. Plant growth-promoting bacteria (PGPB)

They play a pivotal role in enhancing plant drought tolerance through multiple mechanisms, including the production of phytohormones, secretion of hydrolytic enzymes, activity of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, synthesis of exopolysaccharides (EPS), siderophore production, phosphate solubilisation, and emission of volatile compounds like hydrogen cyanide (HCN) and hydrogen sulphide (H_2S).

Indole-3-acetic acid (IAA), a key auxin produced by PGPB, is predominantly concentrated in the shoot apex but plays a crucial role in root development through its polar transport. The IAA synthesized at the shoot tip is transported downward to the roots, where it promotes lateral root formation. This enhanced root architecture enables plants to access deeper soil moisture, improving drought tolerance. Additionally, PGPB-produced gibberellins and cytokinins synergistically stimulate root growth and branching, further aiding plant adaptation under water-limited conditions. For instance, inoculation with *Azospirillum*

brasilense has been shown to enhance root growth in wheat, facilitating better water uptake during drought stress (Pereyra *et al*., 2009)

The secretion of hydrolytic enzymes, including amylases, by PGPB aids in the breakdown of complex carbohydrates in the soil, releasing similar sugars that can be assimilated by plants, thereby supporting energy metabolism under stress conditions. This contributes to improved plant resilience during drought (Fanai *et al*., 2024).

ACC deaminase activity in PGPB lowers plant ethylene levels, a hormone that typically inhibits root growth under stress. By degrading ACC, the precursor to ethylene, these bacteria facilitate sustained root development even in drought conditions. For example, inoculation with ACC deaminase-producing *Pseudomonas* species has been associated with enhanced root length and biomass in drought-stressed plants (Chen *et al*., 2022).

The synthesis of EPS by PGPB contributes to soil aggregation and improved water retention around the root zone, creating a microenvironment that buffers plants against drought-induced desiccation. EPS-producing bacterial strains like *Bacillus amyloliquefaciens* have been reported to enhance soil moisture retention, thereby supporting plant growth under water-limited conditions (Das, 2018).

Siderophore production by PGPB chelates iron from the soil, making it more available to plants. This process not only facilitates iron uptake but also suppresses pathogenic microbes by limiting their access to iron, thereby indirectly supporting plant health during drought stress. Strains such as *Pseudomonas fluorescens* have demonstrated significant siderophore production, enhancing plant growth under adverse conditions (Deka *et al*., 2019).

PGPB also solubilize inorganic phosphate, converting it into forms accessible to plants, which is crucial for energy transfer and metabolic processes, especially under drought stress. *Bacillus* and *Pseudomonas* species have been identified as efficient phosphate solubilizers, promoting buffer growth in drought-affected plants (Bakki *et al*., 2024).

The production of volatile compounds such as HCN and H_2S by certain PGPB strains can induce systemic tolerance in plants, enhancing their defensive mechanism against abiotic stresses, including drought. For instance, H_2S has been implicated in stomata closure regulation, reducing water loss during drought conditions (Lisjak *et al*., 2010).

Collectively, these multifaceted mechanisms employed by PGPB offer a sustainable and eco-friendly approach to enhancing crop resilience in arid and semi-arid regions, thereby contributing to agricultural productivity under drought stress.

2.6. Microbiome-based rhizosphere engineering

Microbiome-based rhizosphere engineering is considered an advanced strategy compared to traditional soil management, breeding, or genetic engineering approaches, as it seeks to enhance plant drought tolerance by manipulating root-associated microbial communities to improve plant resilience under stress conditions. This can be achieved through two major conceptual methodologies: top-down and bottom-up strategies, which differ based on the primary initiator of microbial community modulation.

Bottom-up approaches are externally driven strategies where researchers deliberately introduce selected microbial strains, consortia, or synthetic microbial communities into the rhizosphere. These interventions aim to restructure the existing microbiome to support plant health under stress. For instance, studies have demonstrated that synthetic consortia composed of multiple drought-adaptive bacterial strains can significantly enhance drought tolerance by modulating soil properties, increasing water availability, and promoting stress-responsive plant traits (Fitzpatrick *et al*., 2020). The beneficial mechanisms involved include the microbial production of phytohormones such as indole-3-acetic acid (IAA) and abscisic acid (ABA)—with ABA playing a critical role in inducing stomatal closure to minimize water loss—as well as the accumulation of osmoprotectants like proline and trehalose, and the secretion of exopolysaccharides (EPS) that enhance soil structure and moisture retention.

Top-down approaches, on the other hand, are plant-driven and rely on the plant's intrinsic ability to shape its own rhizosphere microbiome. Under drought stress, plants alter the composition of their root exudates—releasing specific organic acids, sugars, and amino acids—which act as chemical cues or nutrient sources to selectively attract and recruit beneficial microbial taxa. For example, drought-stressed maize plants have been observed to increase the exudation of malic acid, which serves as a chemoattractant for drought-resilient bacteria like *Bacillus velezensis*, enhancing beneficial microbial colonization in the rhizosphere (Yuan *et al*., 2015). This selective recruitment also supports symbiotic associations with arbuscular mycorrhizal fungi (AMF), which improve water uptake and nutrient acquisition, ultimately contributing to greater drought resilience.

Together, top-down and bottom-up strategies represent complementary and sustainable microbiome engineering approaches, offering nature-based solutions to improve crop performance under increasingly water-limited conditions.

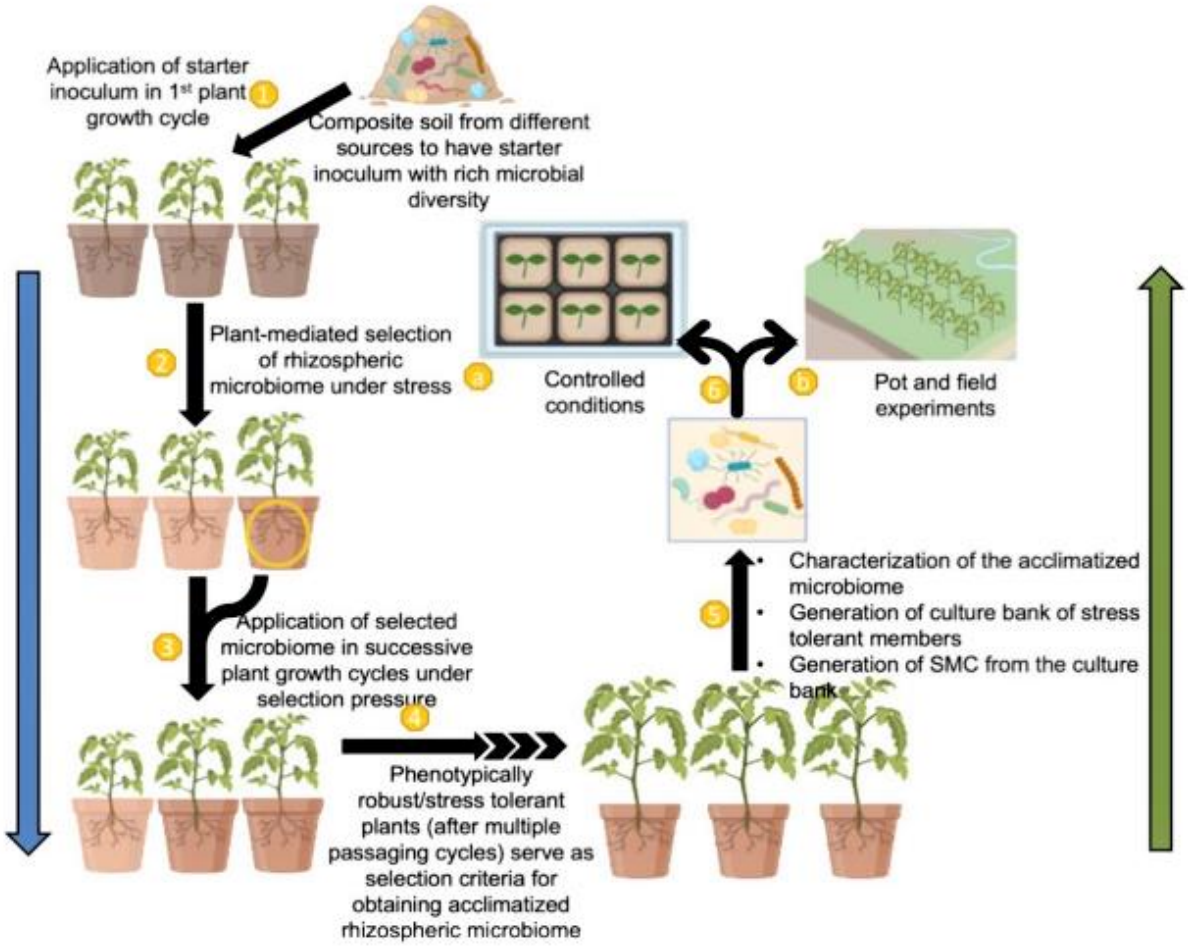

Application of starter inoculum in 1st plant growth cycle
Composite soil from different sources to have starter inoculum with rich microbial diversity
Plant-mediated selection of rhizospheric microbiome under stress
Controlled conditions
Pot and field experiments
Characterization of the acclimatized microbiome
Generation of culture bank of stress tolerant members
Generation of SMC from the culture bank
Application of selected microbiome in successive plant growth cycles under selection pressure
Phenotypically robust/stress tolerant plants (after multiple passaging cycles) serve as selection criteria for obtaining acclimatized rhizospheric microbiome

Figure 1. Combinatorial strategy for engineering the rhizosphere of plants to alleviate various stresses, demonstrating the top-down (blue arrow) and bottom-up (green arrow) approaches (Tyagi *et al*., 2022).

2.7. Research gaps

Although extensive research has explored plant responses to drought stress and the role of plant growth-promoting rhizobacteria (PGPR) in enhancing stress tolerance, there remains limited understanding of how carefully isolated and screened drought-tolerant rhizospheric bacteria, selected based on their compatibility, affect both plant biometric traits and biochemical stress indicators under controlled water deficit conditions.

While this study successfully isolated, screened for PGP traits, and tested the compatibility of rhizospheric bacteria from tomato, it focused on evaluating the individual effects of selected isolates on tomato seedlings under drought stress. However, the formulation and testing of bacterial consortia to assess potential synergistic effects remain an important avenue for future research.

This work therefore advances knowledge on the application of compatible drought-tolerant bacteria in tomato drought resilience, while highlighting the need for further studies on multi-strain consortia to optimize microbial inoculant formulations for sustainable crop production.

3. Objectives

3.1. To study the responses in plant biometrics and stress indicator levels in the presence of water deficit upon treatment with a stress-tolerant soil inoculum.

3.2. To isolate and screen rhizospheric bacteria for plant growth-promoting traits and test their compatibility with each other.

3.3. To analyze the net beneficial effects of shortlisted rhizospheric isolates on tomato seedlings under drought stress.

4. Materials and methods

4.1. Composite soil preparation

As a starter inoculum, a composite soil was constituted for the 0th plant-growth cycle (PGC), by collecting soil from different locations such as arable land, grasslands and nurseries. The composite soil had a loamy texture. The pH and EC of the soil were 7.55±0.03 and 0.015±0.002 dS m^{-1}, respectively.

4.2. Plant growth experiment

A plant growth experiment was initiated using tomato (*Solanum lycopersicum*) seeds of the variety Pusa Sadabahar, procured from the Indian Agriculture Research Institute (IARI) Pusa, New Delhi. The experiment was conducted in a plant growth chamber under controlled conditions, where environmental parameters such as temperature (28±2 ℃), relative humidity (60-70%), and a 16 h light and 8 h dark photoperiod were maintained. The tomato seed were surface sterilized with 0.5% sodium hypochlorite (NaOCl) solution for 1-2 min (Jangir *et al*., 2018), followed by rinsing with autoclaved water 4–5 times, and was sown in two separate seedling trays containing autoclaved and unautoclaved soil for approximately 25 days on before being transplanted to plastic pots, each containing ~300 g soil.

The composite rhizospheric soil sample was used as a starter inoculum where it was applied to both control and drought-stressed tomato plants to initiate microbiome acclimatization. From the 1st PGC onwards, the experiment was expanded to include a total of twelve treatment sets, with six each under autoclaved and unautoclaved soil conditions, to evaluate the influence of live and heat-killed microbiome. The treatment groups under unautoclaved soil were: control (C), control + inoculum (C+I), control + autoclaved inoculum (C+AI), stress (S), stress + inoculum (S+I), stress + autoclaved inoculum (S+AI). Correspondingly, the autoclaved soil treatments were labeled as: autoclaved control (AC), autoclaved control + inoculum (AC+I), autoclaved control + autoclaved inoculum (AC+AI), autoclaved stress (AS), autoclaved stress + inoculum (AS+I), autoclaved stress + autoclaved inoculum (AS+AI).

The drought acclimatization process was achieved by withholding water from designated treatment sets. After transplanting the tomato seedlings to respective pots under unautoclaved and autoclaved soil conditions, all plants were watered uniformly every alternative day for one week to allow initial establishment of the seedlings. Following this, the microbial inoculum, prepared as soil slurry from the rhizosphere of three plants exhibiting the best

phenotypic traits, from C+I, S+I, AC+I and AS+I respectively from previous cycle was applied to the appropriate treatment groups (C+I, C+AI, S+I, S+AI, and their autoclaved counterparts). A volume of 25 ml of this soil slurry was added per plastic pot. Immediately after giving inoculum application, watering was stopped for the stress groups (S, S+I, S+AI, AS, AS+I, AS+AI), while control groups continued to receive measured irrigation of 25 ml every alternate day to maintain well-watered conditions. After maintaining the stress treatment for another 7 days, plant sampling was conducted for physiological and biochemical analysis and rhizospheric soil was collected to prepare inoculum for subsequent cycle. A total of 3 cycles of plant growth were completed in this study.

4.3. Plant growth parameters and quantification of stress markers

The shoot length, root length, shoot fresh and dry weight, root fresh and dry weight, number of branches and leaves were measured. Drought stress markers, viz. proline content, chlorophyll content, carotenoid content, and malondialdehyde (MDA) level were quantified from tomato leaves.

4.3.1. Chlorophyll and carotenoid level estimation: 0.1 g of leaf samples (fresh) was homogenized in 1 ml of 80% acetone. Extracted pigments were estimated by recording the absorbance at 663 and 645 nm for chlorophyll b and a, and at 480 and 510 nm for carotenoid content using a multi-mode microplate reader (SpectraMax® M2^e). Chlorophyll and carotenoid contents were represented in mg per g of fresh weight (Arnon, 1949).

4.3.2. MDA level quantification: MDA quantification was done to measure lipid peroxidation in plants. Fresh leaf tissue (0.5 g) was blended with 1 ml of 0.5% thiobarbituric acid containing 20% trichloroacetic acid. The mixture was heated at 90℃ for 30 min in a water bath (Waiometra) and then allowed to cool down to room temperature. Subsequently, the mixture was centrifuged at 5000 × *g* for 5 min. The absorbance was taken at 400, 532, and 600 nm in a multi-mode microplate reader (SpectraMax® M2^e). The final calculation was done by subtracting the nonspecific absorbance. The concentration of MDA was determined by its molar extinction coefficient (155 nm^{-1} cm^{-1}), and the results were represented in nmol MDA per g fresh weight (Hodges *et al*., 1999)

4.3.3. Proline estimation: The procedure described by Bates *et al*. (1973) with a minor alteration was used to estimate the proline content in the leaves (measured by convention). Fresh leaves (0.1 g) were homogenized in 3% (w/v) sulfosalicylic acid, and then centrifuged at 8500 × *g* for 10 min. 1 ml of supernatant from the centrifuged sample was mixed with 1 ml each of acid-ninhydrin reagent and glacial acetic acid. Then, the mixture was boiled at 100℃

for 1 h in the water bath. The solution was allowed to cool down, followed by the addition of 2 ml of toluene. The mixture was vortexed for 20 s. The phase consisting of toluene was isolated from the fluid phase. The absorbance was noted at 520 nm against a blank (toluene) using a multi-mode microplate reader (SpectraMax® M2^{e}). The proline concentration in the sample concentration in the samples was determined by preparing a standard curve with a known proline concentration.

4.4. Isolation of rhizospheric bacteria

After completion of the 1st PGC, rhizospheric soil samples were collected from the root zones of drought-stressed tomato plants to isolate bacterial communities potentially involved in drought adaptation.

Bacteria were isolated from the soil sample by spread plate technique. 1 g of soil sample was taken in 9 ml of saline (0.9%) solution in a 15 ml centrifuge tube and then it is serially diluted from 10^{-1} to 10^{-3} in micro-centrifuge tubes. From each dilution 0.1 ml of the aliquot was spread onto sterile plates containing two different complex media: nutrient agar (NA) and Luria-Bertani (LBA) agar. Plates were incubated at 30±2℃ for 24 h, and morphologically distinct colonies were selected for further purification and screening.

4.5. Screening of rhizospheric isolates for plant-growth promoting traits

Following isolation, all purified bacterial strains were named and then screened in a stepwise manner to evaluate their plant growth-promoting (PGP) potential under stimulated drought conditions.

As an initial selection criterion, isolates were first assessed for their ability to grow on a tomato root exudate (TRE) mimicking agar medium, reflecting their adaptability to host-specific rhizosphere conditions. Plates were incubated at 28±2℃ for 48–72 h. Only those isolates that showed positive and sustained growth were considered for further screening.

To assess their tolerance to osmotic stress, the selected isolates were then tested for growth on nutrient agar plates supplemented with 35% polyethylene glycol (PEG-6000). PEG is commonly used to simulate drought conditions in vitro by creating osmotic stress without being toxic to microbes. The 35% concentration was chosen to mimic severe water deficit. Growth was evaluated at both half-strength and full-strength PEG concentrations, representing moderate and severe drought stress, respectively. Plates were incubated at 28±2 ℃ for 48–72 h, and growth was evaluated.

Following this, the isolates were further subjected to plant-growth promoting traits.

4.5.1. Exopolysaccharide (EPS) production

Qualitative screening using Congo red agar:

Qualitative assessment of exopolysaccharide (EPS) production by the bacterial isolates was carried out using Congo red agar. The medium was composed of brain heart infusion broth (37 g/l), sucrose (50 g/l), agar (10 g/l), and Congo red dye (0.8 g/l). Congo red was prepared separately as a concentrated aqueous solution and autoclaved at 121°C for 15 min. After sterilization, it was added to the rest of the medium once cooled to approximately 55°C to avoid thermal degradation of the dye. The plates were inoculated with the bacterial cultures and incubated aerobically at 37°C for 24 h. EPS production was indicated by the formation of black colonies with a dry crystalline consistency. Colonies that remained pink and moist were considered non-slime producers, while colonies showing darkening without dry crystalline appearance were classified as indeterminate (Freeman et al., 1989).

EPS production and estimation using phenol-sulfuric acid method:

For quantitative estimation of exopolysaccharide production, bacterial isolates were inoculated into a sterile complex medium containing magnesium sulfate (0.025%), calcium chloride dihydrate (0.009%), potassium chloride (0.05%), sodium bromide (0.006%), peptone (0.5%), yeast extract (0.5%), and glucose (2%), and incubated at 30°C for 7 days under shaking conditions (160 rpm) in a rotary shaker (Chambi *et al*., 2021). After incubation, the bacterial cultures were centrifuged at 13000 × *g* for 20 min to separate the supernatant. EPS present in the supernatant was precipitated by adding chilled absolute ethanol in a 1:2 (supernatant:ethanol) ratio and incubating the mixture at 4°C for 24 h. The precipitated EPS was recovered by centrifugation at 13000 × *g* for 20 min and dried at 40°C.

The dried EPS was then resuspended in 0.5 ml of sterile distilled water, and 0.5 ml of 5% phenol along with 2.5 ml of concentrated sulfuric acid was added. After thorough mixing, the solution was incubated, and the absorbance was recorded at 640 nm against a blank to estimate the total polysaccharide content using the phenol-sulfuric acid method.

4.5.2. Indole-3-Acetic Acid (IAA) production

IAA production and estimation:

Quantitative estimation of indole-3-acetic acid (IAA) production by bacterial isolates was performed using the colorimetric method involving Salkowski's reagent. Freshly grown bacterial cultures were inoculated into nutrient broth supplemented with 1% sterile-filtered L-tryptophan as a precursor for IAA biosynthesis. Specifically, 100 µl of an active bacterial culture was added to 5 ml of L-tryptophan-amended nutrient broth and incubated at 30°C with shaking at 150 rpm for 48 h. After incubation, cultures were centrifuged at 7500 rpm for

15 min, and the cell-free supernatant was collected. For IAA detection, 2 ml of supernatant was mixed with 5 ml of freshly prepared Salkowski's reagent (prepared by combining 2 ml of 0.5 M $FeCl_3$ solution, 30 ml of 70% perchloric acid, and 68 ml of distilled water). A few drops of ortho-phosphoric acid were also added to enhance color development. The reaction mixture was incubated in the dark at room temperature for 30 min, leading to the formation of a pink to reddish color indicative of IAA production. The absorbance of the developed color was measured at 530 nm using a spectrophotometer. The concentration of IAA produced by the isolates was determined by extrapolating the absorbance values against a standard curve generated using known concentrations of pure IAA.

4.5.3. ACC Deaminase Activity Assay

Qualitative screening:

The ability of bacterial isolates to utilize 1-aminocyclopropane-1-carboxylate (ACC) as a sole nitrogen source was assessed on a Dworkin and Foster (DF) minimal salt medium. The DF medium was prepared using components such as KH_2PO_4 (4 g/l), Na_2HPO_4 (6 g/l), $MgSO_4 \cdot 7H_2O$ (0.2 g/l), glucose (2 g/l), K-salt of gluconic acid (2 g/l), and citric acid (2 g/l), along with micronutrients including $FeSO_4$, H_3BO_3, $MnSO_4$, $ZnSO_4$, $CuSO_4$, and MoO_3. After sterilization, sterile-filtered After sterilization, 100 µL of sterile-filtered 0.3 M ACC solution was spread onto the cooled DF agar plates, followed by spot inoculation of the bacterial isolates. Plates were incubated at 37°C for 72 h. Positive growth on ACC-amended DF medium indicated ACC deaminase activity.

Quantitative estimation:

For quantitative estimation, bacterial isolates were initially grown in tryptic soy broth (TSB) at 28°C overnight. Cells were harvested by centrifugation at 8500 rpm for 10 min at 4°C, washed, and resuspended in DF medium without a nitrogen source. After overnight incubation, the cultures were again centrifuged and the pellets were washed with 0.1 M Tris-HCl buffer (pH 7.6). The cell pellet was finally resuspended in 0.1 M Tris-HCl buffer (pH 8.5) and divided into three aliquots. Two aliquots were used for ACC deaminase assay and one for protein estimation. For assay, 30 µl toluene was added to permeabilize the cells, followed by the addition of 20 µl of 0.5 M ACC to 200 µl of permeabilized cells. After incubation at 30°C for 30 min, 0.56 M HCl was added to stop the reaction. Samples were centrifuged at 14000 × *g*, and the supernatant was reacted with 2,4-dinitrophenylhydrazine (2,4-DNP) in acid solution, followed by addition of 2 M NaOH. The development of color was measured at 540 nm using a spectrophotometer. Blanks without cells were included to calibrate the measurements (Penrose and Glick, 2003).

4.5.4. Phosphate Solubilization Assay

Qualitative screening using NBRIP agar plates:

Phosphate solubilization by bacterial isolates was qualitatively assessed using National Botanical Research Institute's Phosphate (NBRIP) medium. The medium was composed of glucose (10 g/l), $MgCl_2 \cdot 6H_2O$ (5 g/l), $MgSO_4 \cdot 7H_2O$ (0.25 g/l), KCl (0.2 g/l), $(NH_4)_2SO_4$ (0.1 g/l), $Ca_3(PO_4)_2$ (5 g/l), and bromophenol blue (0.025 g/l) as an indicator dye. The medium was adjusted to pH 7 before autoclaving. Bacterial isolates were spot-inoculated onto NBRIP agar plates and incubated at 30°C for 5–7 days. Positive phosphate solubilization was indicated by the formation of a clear halo zone around the bacterial colonies against the blue background (Nautiyal, 1999).

Quantitative estimation:

For quantitative analysis, bacterial isolates were first grown in tryptic soy broth (TSB) for 24 h at 28°C. After incubation, 1 ml of the bacterial suspension was transferred into 25 ml of sterile NBRIP broth containing the same components as the agar medium. Cultures were incubated for 5 days at 28°C under shaking conditions at 130 rpm. After incubation, the broth cultures were centrifuged at 13000 × *g* for 10 min, and the clear supernatant was collected for analysis. Quantification of solubilized phosphate was performed using the molybdenum blue method (Murphy and Riley, 1962). To 1 ml of the supernatant, 0.5 ml of 5.54×10^{-2} M ammonium molybdate and 0.5 ml of 0.25 N sulfuric acid were added, followed by 1 ml of 2 M ascorbic acid. The absorbance was measured at 880 nm against a reagent blank, and the amount of phosphate solubilized was determined using a standard curve prepared with KH_2PO_4 (Nautiyal, 1999).

4.5.5. Siderophore Production Assay

Qualitative screening using CAS agar method:

Qualitative detection of siderophore production was carried out using Chrome Azurol S (CAS) medium. The CAS reagent was prepared separately by dissolving 121 mg of CAS dye in 100 ml of distilled water, 729 mg of HDTMA in 400 ml distilled water, and 10 mM ferric chloride solution prepared by dissolving 1 mM $FeCl_3 \cdot 6H_2O$ in 10 mM HCl. These solutions were mixed with sterile nutrient media while cooling at 55°C to prepare CAS agar. Bacterial isolates were spot-inoculated onto CAS agar plates and incubated at 30°C for 3–5 days. Siderophore production was indicated by the formation of an orange or yellow halo zone around the bacterial colony against the blue background of the medium.

Quantitative estimation by microplate assay:

Quantitative estimation of siderophore production was performed using a modified microplate method. Freshly grown bacterial cultures (25 µl) were added to 2.5 ml of LB broth in microcentrifuge tubes and incubated at 28°C for 48 h. After incubation, cultures were centrifuged at 10,000 rpm for 10 min, and 100 µl of the cell-free supernatant was transferred to a microplate well. An equal volume (100 µl) of CAS reagent was added to each well and incubated briefly. Absorbance was measured at 630 nm using a microplate spectrophotometer. Siderophore production was quantified using the formula:

$$Siderophore\ production\ (\%) = \frac{(Ar - As)}{Ar} \times 100$$

where *Ar* is the absorbance of the reference (uninoculated CAS reagent) and *As* is the absorbance of the sample (supernatant mixed with CAS reagent).

In addition to plant-growth promoting properties, the ability of isolates to form biofilms was assessed as it plays a crucial role in enhancing microbial colonization and survival under drought conditions (Arora and Verma, 2017).

4.5.6. Biofilm formation and quantification

The biofilm formation ability of the selected isolates was evaluated under drought-simulated conditions using a microtiter plate assay adapted from O'Toole and Kolter (1998), with modifications. Fresh overnight bacterial cultures grown in nutrient broth (NB) at 30°C were diluted in a 1:100 ratio into fresh NB medium supplemented with 35% PEG-6000. A volume of 100 µl of the diluted culture was transferred into individual wells of sterile 96-well polystyrene microtiter plates. The plates were incubated at 30°C for 48 h under static conditions to allow biofilm formation. After incubation, the planktonic cells were gently removed, and the wells were washed twice with sterile milli-Q water to remove unattached cells. Biofilms were stained by adding 0.1% crystal violet solution to each well and incubating for 30 min. Excess stain was washed off with sterile water, and 95% ethanol was added to each well to solubilize the bound dye. The absorbance was measured at 590 nm using a multi-mode microplate reader (SpectraMax® M2^{e}). Blank wells containing only uninoculated NB medium were used for baseline correction.

4.6. Compatibility testing of isolates

To assess the compatibility of the selected bacterial isolates for potential consortium development, a cross-streaking method was employed. Each isolate was streaked perpendicularly against the others on nutrient agar (NA) plates and incubated at 30°C for 24 h. The growth of isolates at the intersection points was observed. Compatibility was indicated

by uninterrupted growth of both isolates without any clear zone of inhibition at the crossing points, whereas the presence of an inhibition zone suggested antagonism.

4.7. Bacterial genomic DNA isolation and 16S rRNA gene amplification

4.7.1. Bacterial genomic DNA isolation:

Genomic DNA was extracted from bacterial isolates cultured overnight in NB medium by inoculating from a single colony. Approximately 1.5 ml of overnight culture was harvested by centrifugation at 7500 rpm for 10 min. The supernatant was discarded, and the pellet was resuspended in 567 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Next, 30 µl of 10% SDS and 10 µl of Proteinase K (20 mg/ml) were added to lyse the cells, and the mixture was incubated at 37°C for 1 h. Following lysis, 80 µl of pre-warmed CTAB-NaCl solution (cetyltrimethylammonium bromide at 10% w/v with 0.7 M NaCl) was added to the lysate. The mixture was incubated at 65°C for 10–30 min, or until the solution became clear. After cooling to room temperature, 5 µl of RNase was added, and the sample was incubated at 37°C for an additional 20–30 min to remove RNA contamination. An equal volume of chloroform:isoamyl alcohol (24:1) was added, mixed gently, and centrifuged at 14000 rpm for 12 min at 4°C. The upper aqueous layer was carefully transferred to a new tube, and an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to further purify the DNA. This mixture was centrifuged again at 14,000 rpm for 20 min at 4°C. The upper phase was transferred to a new tube, and 0.6 volumes of chilled isopropanol were added to precipitate the DNA. For higher yield, the samples were stored at –20°C overnight.The DNA was pelleted by centrifugation at 14,000 rpm for 20 min at 4°C. The supernatant was discarded, and the pellet was washed with 1 ml of chilled 70% ethanol. After repeating the wash step once more, the pellet was air-dried at 55°C for 5–10 min (avoid over-drying). Finally, the dried DNA pellet was resuspended in 50–100 µl of TE buffer depending on pellet size and stored at 4°C for short-term use or -20°C for long-term storage (Wilson, 2001).

4.7.2. 16S rRNA gene amplification via polymerase chain reaction (PCR)

The purified genomic DNA was used as a template for 16S rRNA gene amplification using standard primers. Each PCR reaction (total volume: 10 µl) consisted of 5 µl of 2X PCR Master Mix, 0.5 µl each of forward and reverse primers (10 µM), 1 µl of template DNA (60–100 ng), and nuclease-free water to make up the final volume. The PCR conditions were as follows: initial denaturation at 95°C for 5 min; followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 90 s; with a final extension at 72°C for 10 min. The PCR products were verified by agarose gel electrophoresis. A 1.5%

agarose gel was prepared using 1X TAE buffer, and 10 µl of each PCR product was loaded along with a DNA ladder. The gel was run at 90 V for 40 min and visualized under a gel documentation unit. Negative controls (no-template) were also run to ensure no contamination occurred.

4.8. Experimental setup for EPS-treated tomato seedling growth under drought stress

Tomato seeds were surface sterilized using sodium hypochlorite solution, followed by multiple rinses with sterile distilled water to remove residual disinfectant. For plate setup, half-strength water agar (20 g/l) was poured into sterile Petri dishes and allowed to solidify. For stress and EPS-treated conditions, a 20% polyethylene glycol (PEG)-6000) solution was overlaid onto the solidified agar and left overnight to ensure adequate absorption. PEG creates an osmotic gradient, lowering water availability without being toxic to seeds, thereby mimicking water deficit stress. the 20% concentration was chosen to simulate moderate drought conditions. The next day, excess unabsorbed PEG solution was carefully removed prior to seed placement. In the control and stress conditions, sterilized seeds were directly placed on the agar surface (nine seeds per plate). In the EPS-treated condition, a seed priming approach was used: sterilized seeds were incubated in an overnight-grown bacterial suspension (adjusted to OD_{600} = 1.0) for three hours before sowing All plates were incubated at 30°C under a controlled photoperiod (light/dark cycle as per the experimental design) for 9–12 days to allow seedling establishment. Following the incubation period, leaf and root samples were harvested from selected treatments for further morphological traits analysis.

4.9. Statistical analysis

Statistical analysis was performed using R software. All plant growth parameters, stress marker analyses, and quantitative PGP trait data were analysed using one-way ANOVA. For biofilm quantification, a paired t-test was conducted. Following ANOVA, tukey's honestly significant difference (HSD) post-hoc test was applied to determine statistically significant differences between treatment groups.

5. Results

This study employed a multi-passaging approach to investigate the role of a stress-adapted rhizosphere microbiome in mitigating drought stress across successive growth cycles in tomato (*Solanum lycopersicum)*. The experimental design involved growing tomato plants under both control and drought stress conditions across three completed plant growth cycles (PGCs). A specific microbial inoculum, derived from native rhizospheric bacteria, was selected to assess its impact on plant physiological and biochemical responses under stress.

To evaluate the influence of live microbial activity, two independent experimental sets were maintained throughout the study: one comprising unautoclaved soil containing active microbiota, and the other using autoclaved soil to eliminate live microbial influence. Each set included treatments representing various combinations of stress, inoculum, and their respective controls.

Effects of acclimatized microbiome on morphological parameters of plant under drought stress

The third cycle of the experiment was assessed to evaluate the influence of microbiome acclimatization on key morphological parameters of tomato plants subjected to drought stress. Morphological attributes such as shoot length, root length, and the fresh and dry weights of both shoot and root tissues were measured to understand the plant's physical response under varying soil microbial conditions. These parameters serve as reliable indicators of overall plant vigor and drought adaptability. Observations were drawn from two experimental sets: one containing live, unautoclaved microbial communities, and the other using sterilized, autoclaved soil to eliminate active microbial activity. Comparative analysis within each set was performed to determine the effect of drought stress alone and in combination with microbial inoculation, providing insights into the potential of acclimatized microbiomes in enhancing plant resilience.

Effects of acclimatization of microbiome on stress markers levels in plants

After the third round of the multi-passaging experiment, stress marker levels were measured in tomato plants to assess the physiological responses to drought and the potential mitigation effect conferred by microbiome acclimatization.

In both autoclaved and unautoclaved experimental sets, proline accumulation patterns reflected distinct responses to drought-induced osmotic stress. In the autoclaved set, the Stress (AS) treatment recorded the highest proline levels at approximately 15.1 µmol g^{-1} FW,

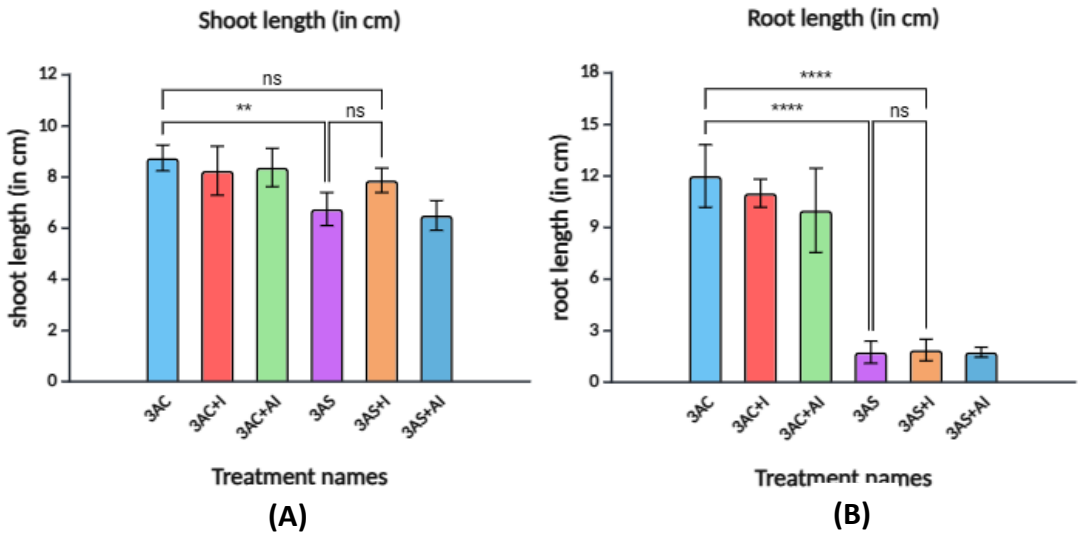
significantly higher than the Control (AC) treatment, which showed 1.40 µmol g^{-1} FW. The Stress + Inoculum (AS+I) treatment exhibited a moderate reduction in proline content to 3.97 µmol g^{-1} FW compared to the Stress group, suggesting partial mitigation of drought effects, potentially due to the applied microbial inoculum. Although the Stress + Inoculum (AS+I) treatment showed higher proline levels compared to Control (AC), the difference was not statistically significant. Similarly, in the unautoclaved set, the Stress (S) treatment showed elevated proline levels of around 15.7 µmol g^{-1} FW, whereas the Control (C) had a much lower concentration at 0.65 µmol g^{-1} FW. The Stress + Inoculum (S+I) treatment also demonstrated a reduction in proline content to 5.04 µmol g^{-1} FW, which was more pronounced than in the autoclaved set. The proline content in Stress + Inoculum (S+I) was significantly higher than Control (C), indicating a measurable osmotic adjustment in response to stress-mediated microbial influence. These differences underscored the enhanced drought-alleviating effect of the acclimatized native microbiome through successive passaging.

A similar trend was observed in malondialdehyde (MDA) levels, an indicator of lipid peroxidation and membrane damage under oxidative stress. In the autoclaved set, the Stress (AS) treatment exhibited the highest MDA content at approximately 0.87 nmol g^{-1} FW, significantly greater than the Control (AC), which recorded 0.27 nmol g^{-1} FW. The Stress + Inoculum (AS+I) treatment showed a reduction in MDA levels to 0.43 nmol g^{-1} FW, suggesting a moderate decrease in oxidative stress symptoms due to the presence of the inoculum. The MDA content in Stress + Inoculum (AS+I) was significantly higher than Control (AC), suggesting enhaced lipid peroxidation under induced stress conditions. In the unautoclaved set, the Stress (S) treatment showed elevated MDA levels of around 0.33 nmol g^{-1} FW, but less than the Control (C), 0.38 nmol g^{-1} FW. The Stress + Inoculum (S+I) treatment demonstrated a more substantial decline to 0.22 nmol g^{-1} FW, reinforcing the protective role of the native, acclimatized microbiome in alleviating oxidative membrane damage. The MDA content in Stress + Inoculum (S+I) was significantly higher than Control (C), suggesting enhanced lipid peroxidation under induced stress conditions. These differences underscored the enhanced drought-alleviating effect of the acclimatized native microbiome through successive passaging.

Chlorophyll and carotenoid contents, which serve as critical indicators of photosynthetic capacity and plant health under stress, exhibited discernible patterns across treatments. In both autoclaved and unautoclaved experimental sets, the Control (AC and C) treatments consistently showed slightly higher levels of chlorophyll a, chlorophyll b, total chlorophyll,

and carotenoids compared to the Stress (AS and S) treatments; however, these differences were not statistically significant ($p > 0.05$). Notably, in both sets, the Stress + Inoculum (AS+I and S+I) treatments recorded significantly higher pigment levels than the Stress-only groups ($p < 0.05$), suggesting a restorative effect of the microbiome on the photosynthetic machinery. This trend was consistent across all four pigment parameters, reflecting the potential of the microbiome to mitigate drought-induced chlorophyll degradation and support plant resilience.

Figure 2. The image shows tomato plants after the third growth cycle from the experimental set with autoclaved soil, grown under controlled and drought stress conditions. Visual differences in plant growth and health are evident between the treatments, with the Stress + Inoculum (S + I) showing improved growth compared to the Stress (S), indicating the potential role of the microbiome in alleviating drought stress. The plants were subjected to stress conditions for 7 days, after adding the microbiome followed by sampling for further analysis.



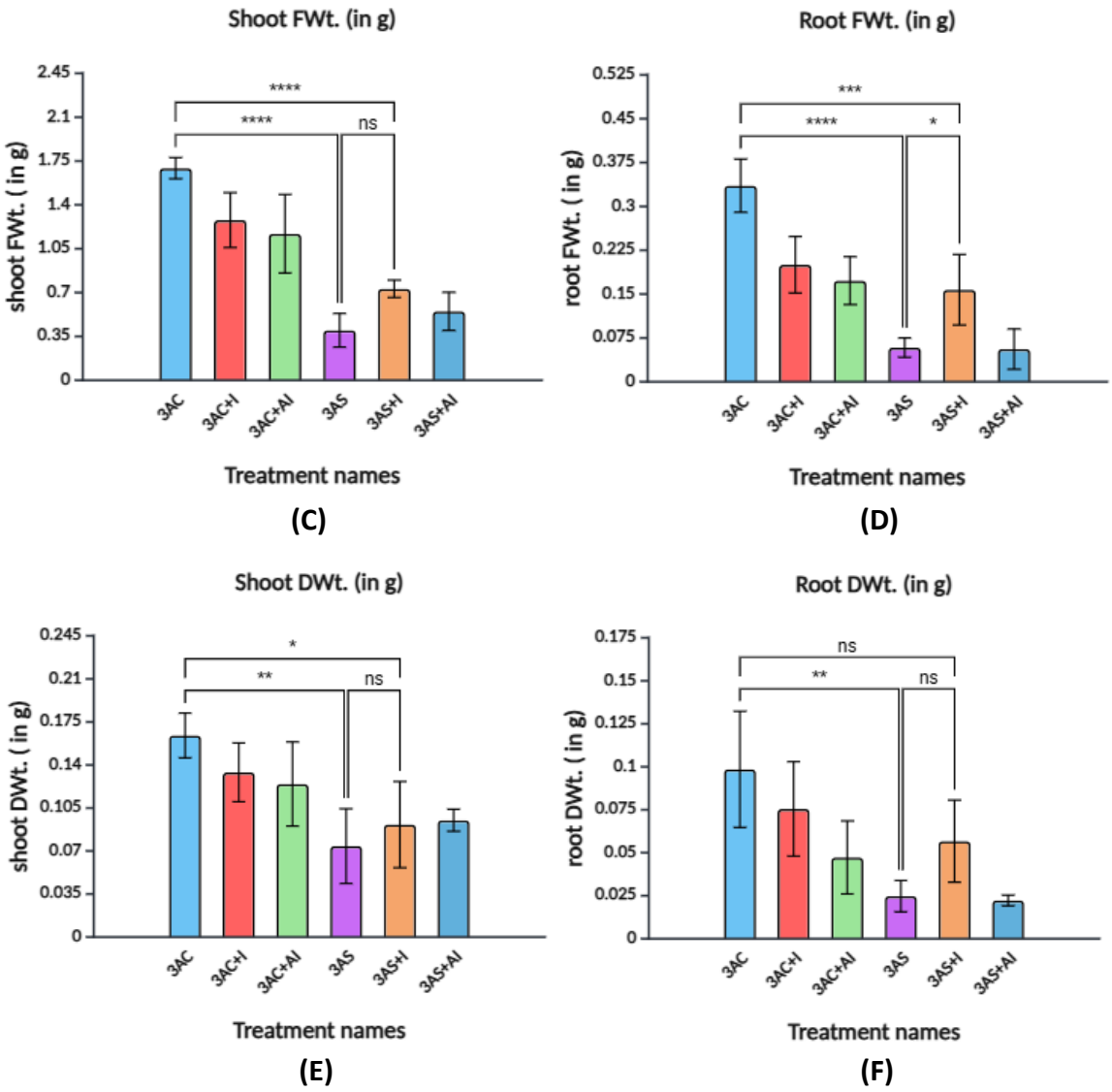
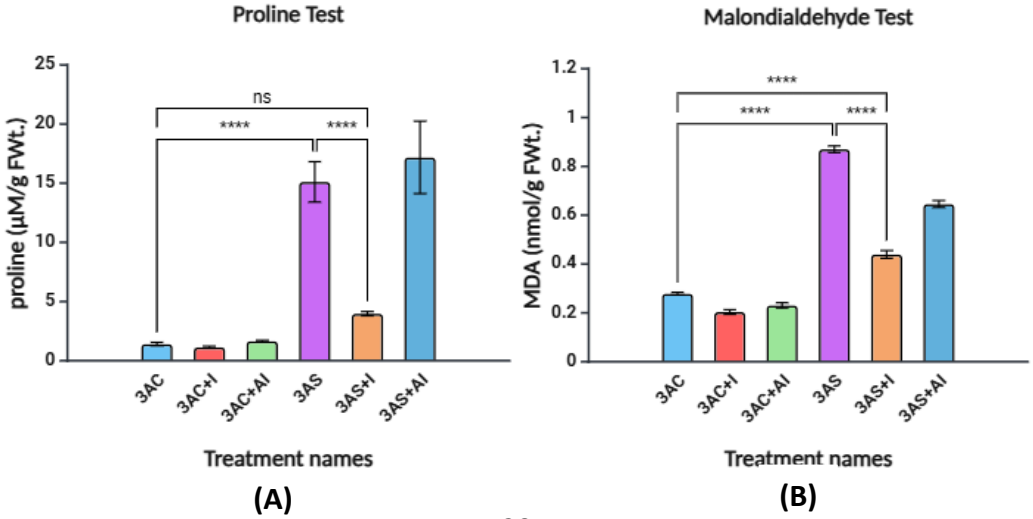


Figure 3. Plant biometrics after the third growth cycle from the experimental set with autoclaved soil, (A) shoot length, (B) root length, (C) shoot fresh weight., (D) root fresh weight., (E) shoot dry weight., and (F) root dry weight. Error bars represents standard deviation within a set of plants (n = 3). *indicates statistical significance difference (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). "ns" indicates not significant.



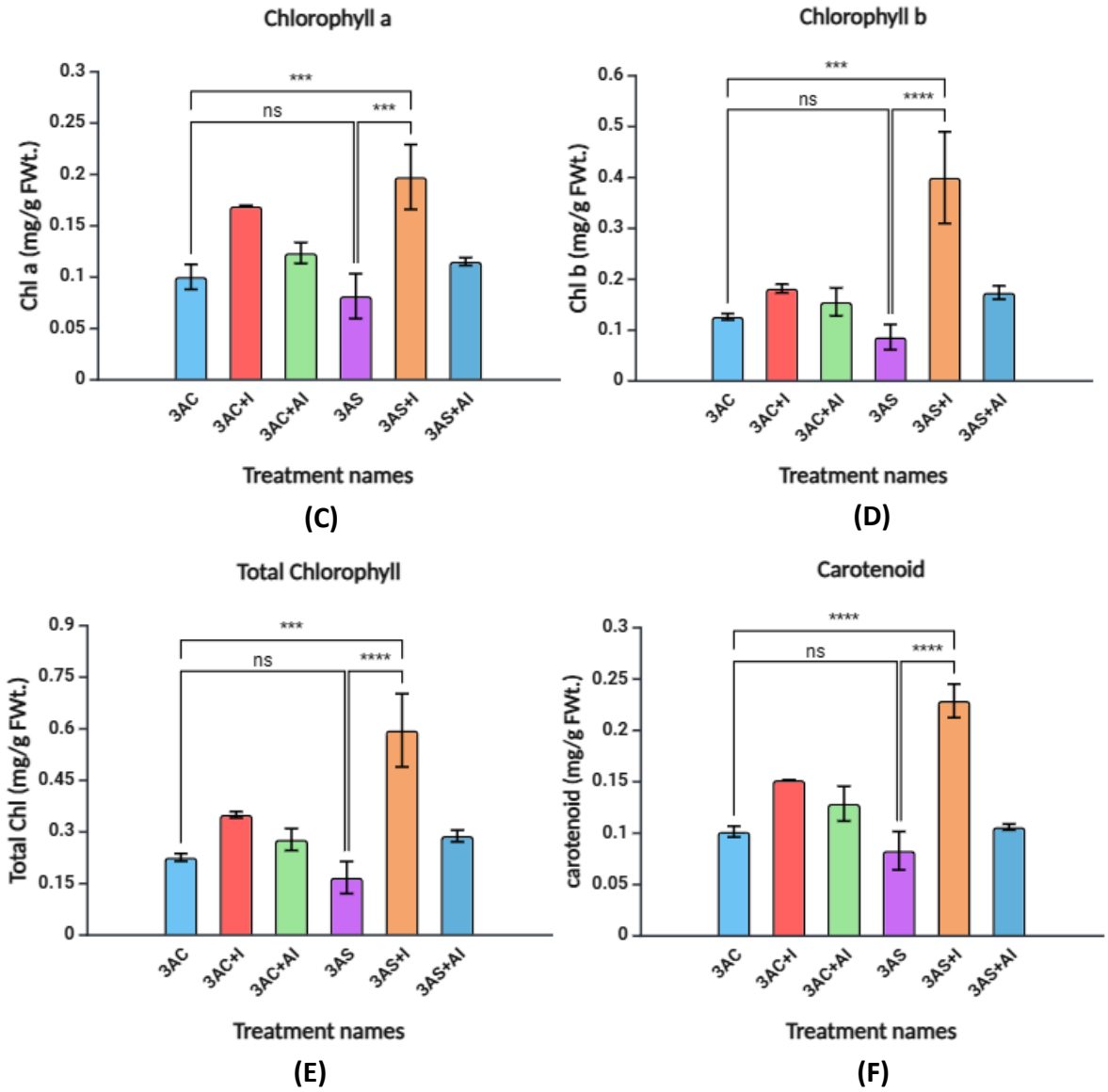


Figure 4. Plant stress marker levels after third growth cycle from the experimental set with autoclaved soil, (A) proline level, (B) malondialdehyde (MDA) level, (C) chlorophyll a, (D) chlorophyll b, (E) total chlorophyll, and (F) carotenoids. Here, error bars represent standard deviation within a set of plants (n = 3). *indicates statistical significance difference (***$p < 0.001$, ****$p < 0.0001$). "ns" indicates not significant.

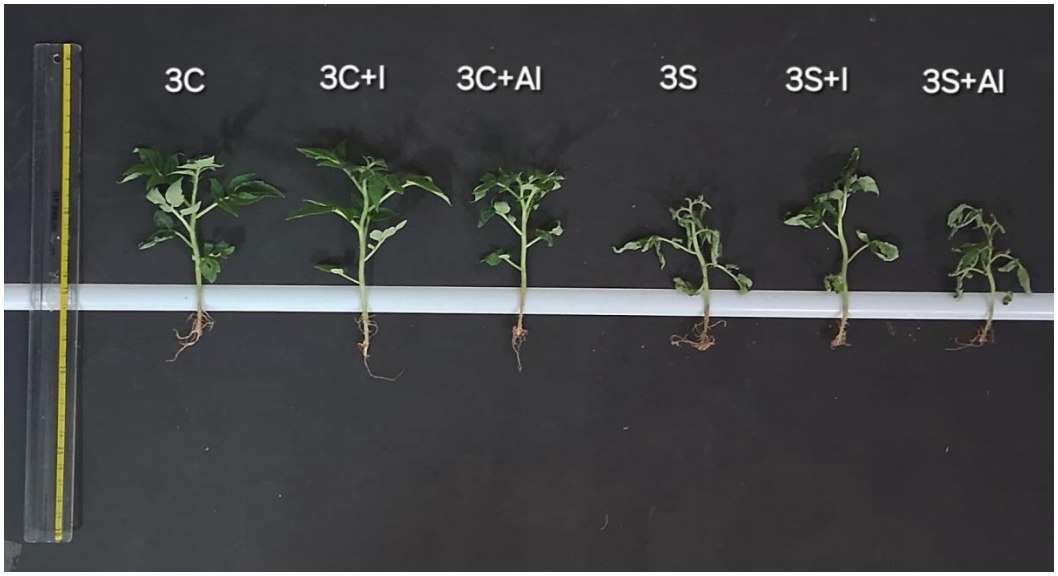
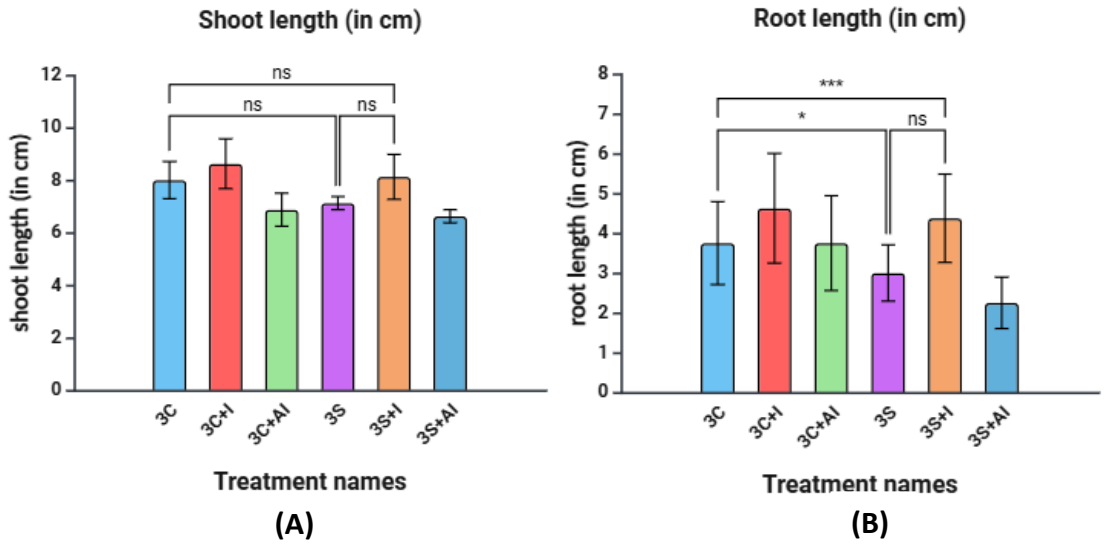


Figure 5. The image shows tomato plants after the third growth cycle from the experimental set with unautoclaved soil, grown under controlled and drought stress conditions. Visual differences in plant growth and health are evident between the treatments, with the Stress + Inoculum (S + I) showing improved growth compared to the Stress (S), indicating the potential role of the microbiome in alleviating drought stress. The plants were subjected to stress conditions for 7 days, after adding the microbiome followed by sampling for further analysis.



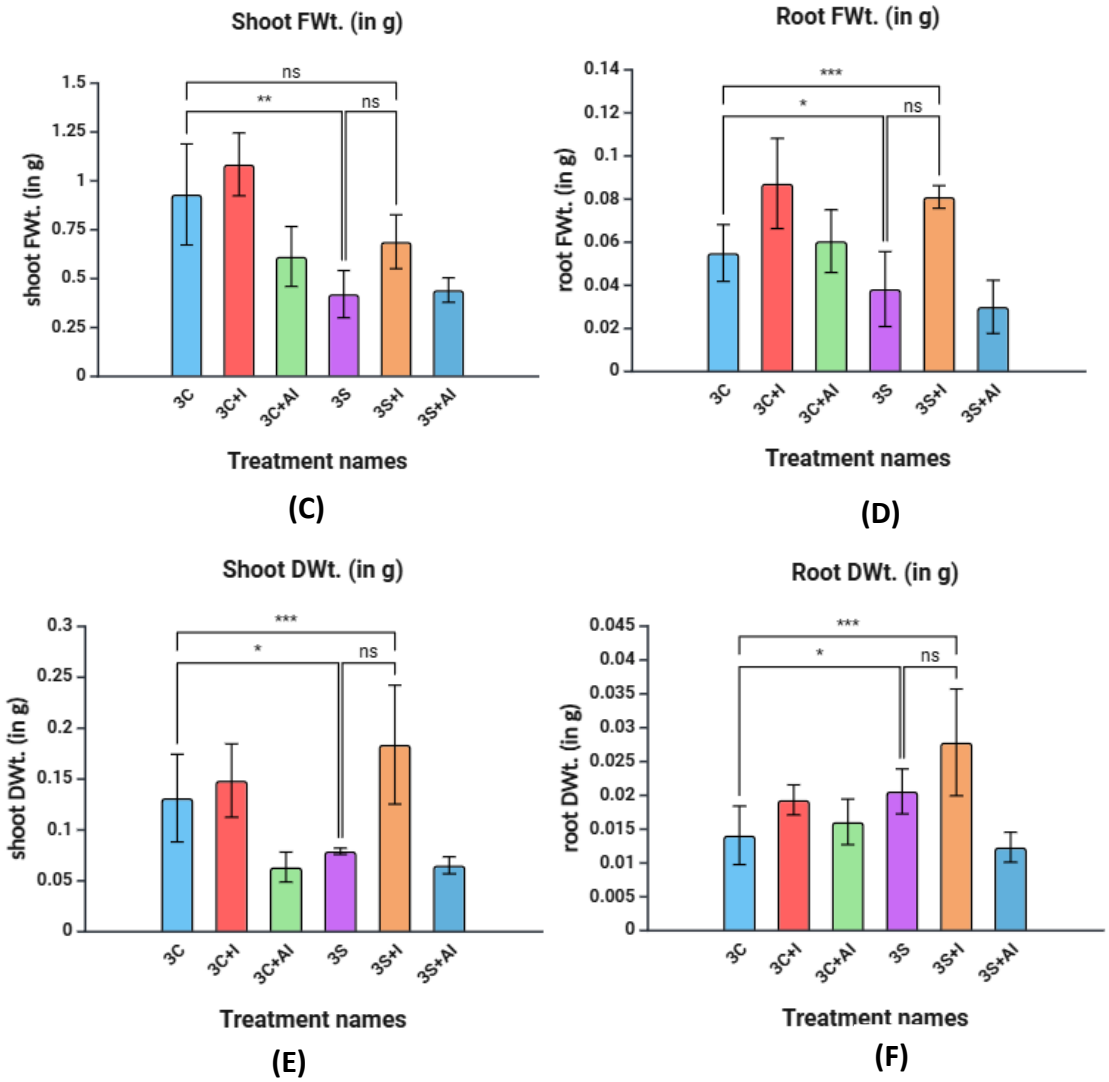
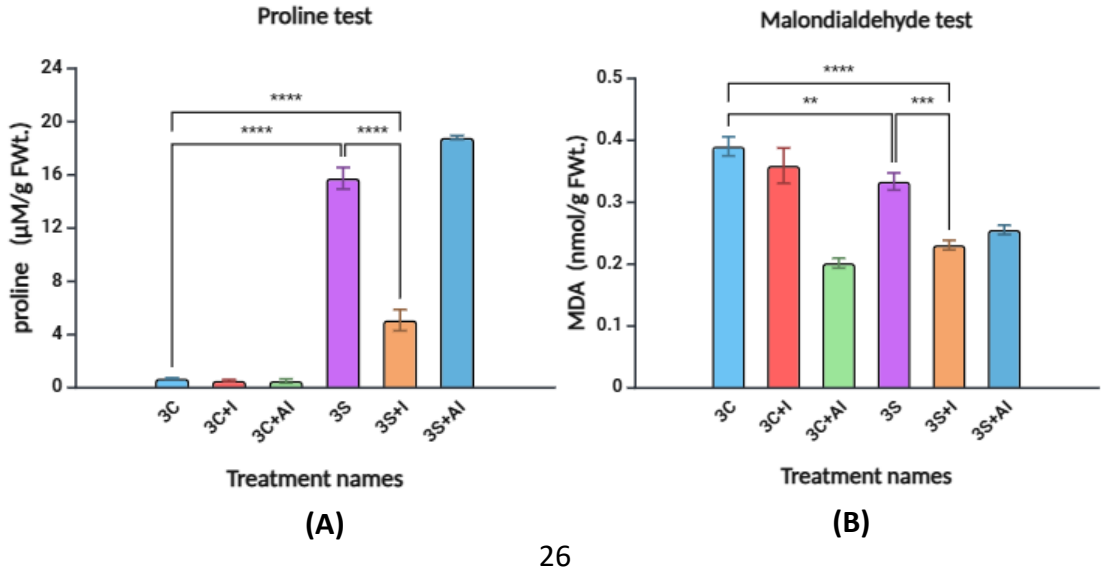


Figure 6. Plant biometrics after the third growth cycle from the experimental set with unautoclaved soil, (A) shoot length, (B) root length, (C) shoot fresh weight., (D) root fresh weight., (E) shoot dry weight., and (F) root dry weight. Error bars represents standard deviation within a set of plants (n = 3). *indicates statistical significance difference (*p < 0.05, **p < 0.01, ***p < 0.001). “ns” indicates not significant.



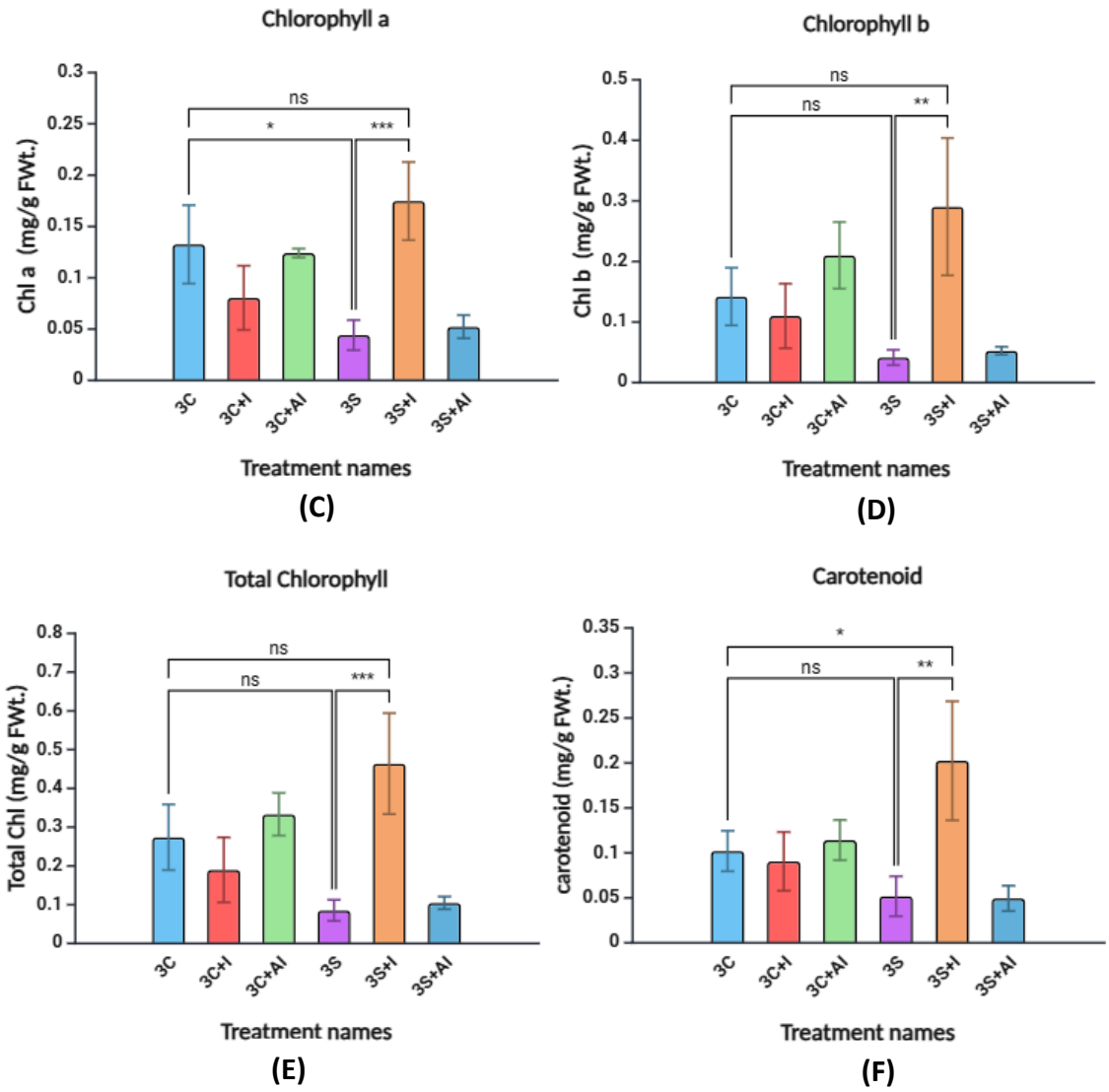
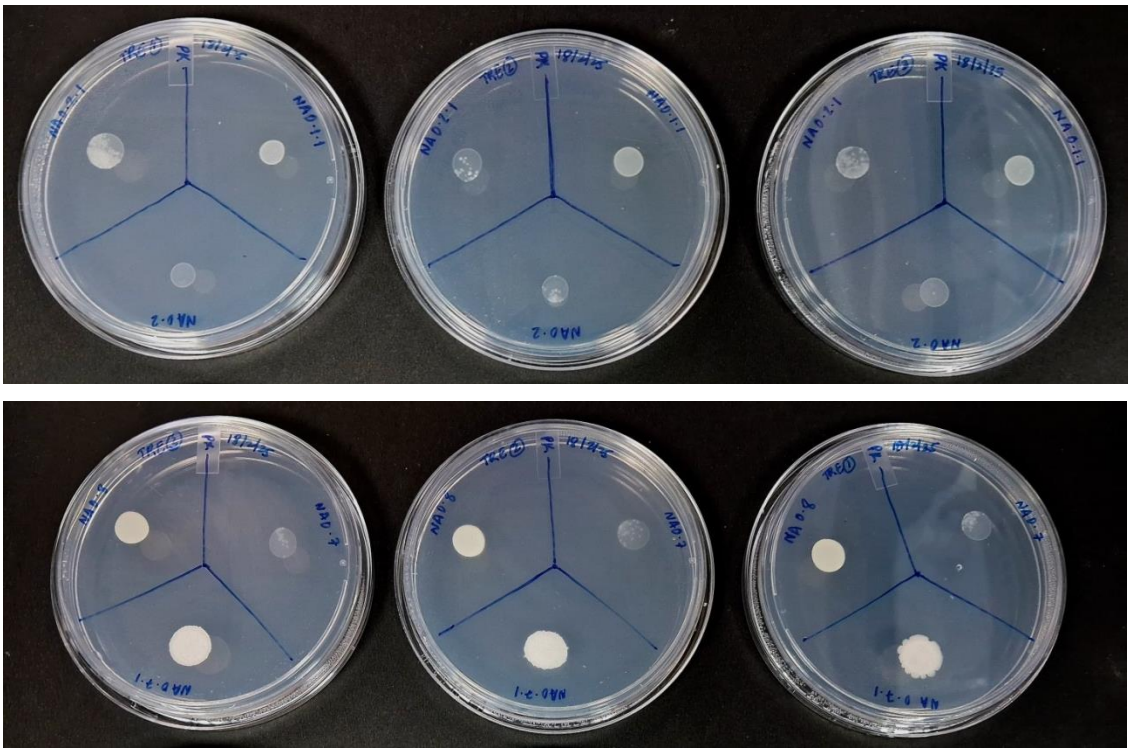


Figure 7. Plant stress marker levels after third growth cycle from the experimental set with autoclaved soil,, (A) proline level, (B) malondialdehyde (MDA) level, (C) chlorophyll a, (D) chlorophyll b, (E) total chlorophyll, and (F) carotenoids. Here, error bars represent standard deviation within a set of plants (n = 3). *indicates statistical significance difference (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). “ns” indicates not significant.

Preliminary screening of drought-tolerant rhizospheric bacteria

Following the completion of the first plant growth cycle (1PGC), rhizospheric soil samples were collected from the autoclaved stress + inoculum (AS+I) for the isolation of drought-adapted bacterial communities. A total of ten morphologically distinct bacterial isolates, designated as PK1, PK2, PK3, PK4, PK5, PK6, PK7, PK8, PK9 and PK10, were obtained by serial dilution and plating on nutrient agar (NA) and Luria-Bertani agar (LBA).

All isolates were initially screened for their ability to grow on tomato root exudate (TRE) mimicking medium to assess host-specific adaptability. Out of the ten isolates, seven isolates (PK1, PK3, PK5, PK6, PK7, PK8, and PK10) demonstrated robust growth and were selected for further evaluation (Figure 8). Subsequently, these seven isolates were tested for drought tolerance on NA and LBA plates at half-strength and full-strength concentrations supplemented with polyethylene glycol-6000 (35% w/v). Based on their growth patterns under PEG-induced osmotic stress, the isolates were classified into low and moderate-to-high drought-tolerant groups (Figure 9). However, all seven isolates were retained for detailed screening of their plant growth-promoting (PGP) traits.



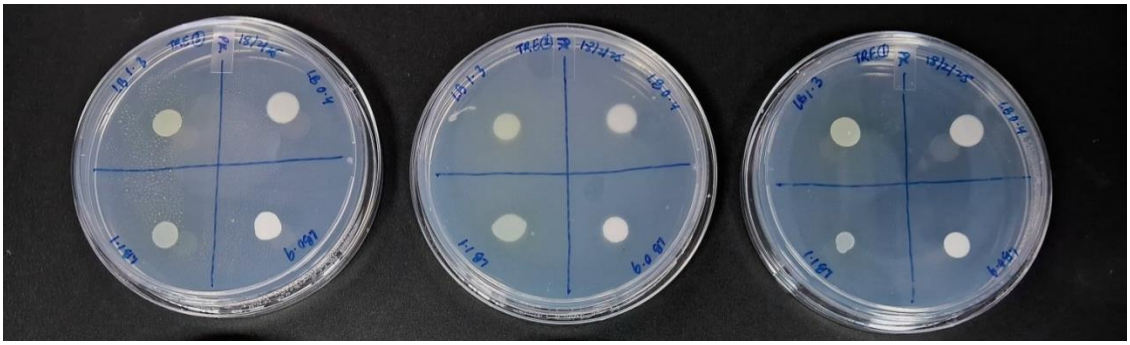


Figure 8. Petri plates containing tomato root exudate (TRE) medium inoculated with ten bacterial isolates.

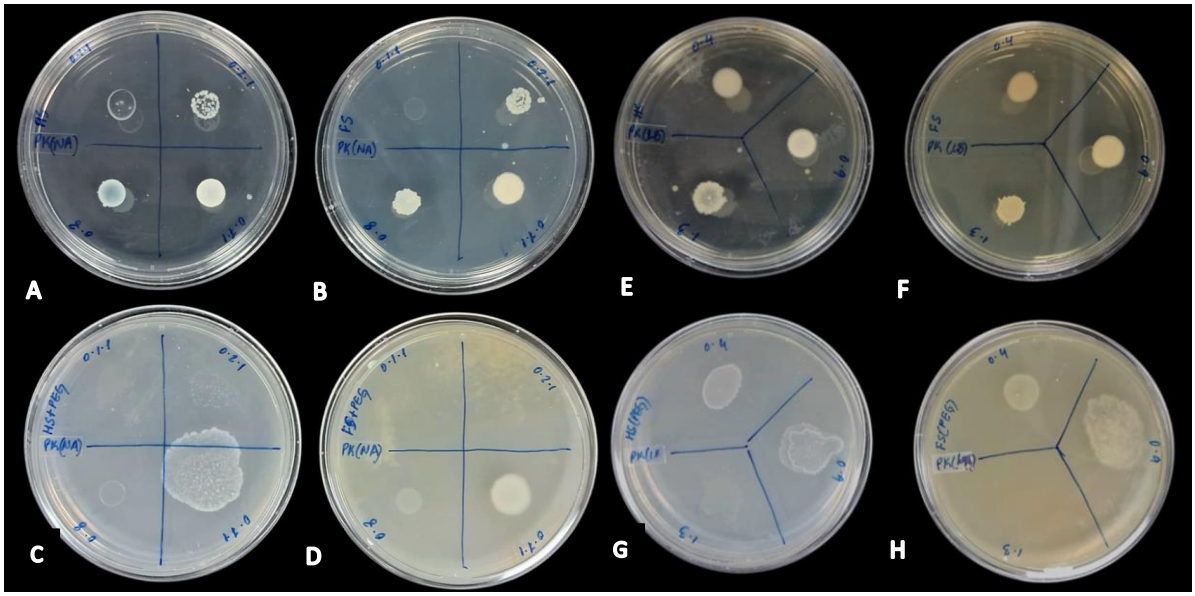


Figure 9. Growth patterns of seven bacterial isolates on nutrient agar (NA) and Luria-Bertani agar (LBA) supplemented with 35% PEG-6000 after 48 h of incubation. (A) half strength NA medium, (B) full strength NA medium, (C) half strength NA medium + PEG, (D) full strength NA medium + PEG, (E) half strength LBA medium, (F) full strength LBA medium, (G) half strength LBA medium + PEG, (H) full strength LBA medium + PEG.

Table 1. Growth and drought tolerance of bacterial isolates on half-strengthas well as full-strength nutrient agar (NA) and LBA supplemented with 35% polyethylene glycol (PEG) 6000.

Bacterial strain	PEG tolerance
PK1	Low
PK3	Low
PK5	Moderate
PK6	Moderate
PK7	Moderate
PK8	Moderate
PK10	Low

Qualitative screening of plant growth-promoting (PGP) traits

The seven drought-tolerant bacterial isolates (PK1, PK3, PK5, PK6, PK7, PK8, and PK10) were evaluated for key plant growth-promoting traits to assess their potential role in enhancing tomato growth under water-deficit conditions.

Exopolysaccharide (EPS) production was assessed using Congo red agar plates, where EPS-producing colonies appear black due to Congo red binding to secreted polysaccharides. A representative image has been included to illustrate the visual screening approach (Figure 10).

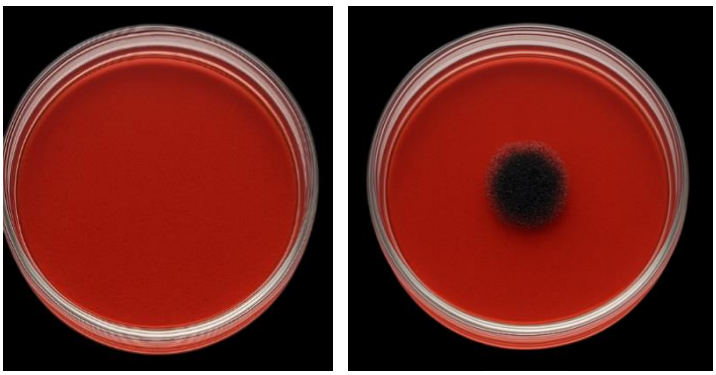


Figure 10. Screening of bacterial isolates for exopolysaccharide (EPS) production on Congo red agar plates. (A) Control plate, (B) EPS-positive plate

Indole-3-acetic acid (IAA) production, a critical phytohormone for root elongation, was detected using Salkowski's reagent, where a pink coloration indicates IAA presence. A representative image has been included to illustrate the visual screening approach (Figure 11).

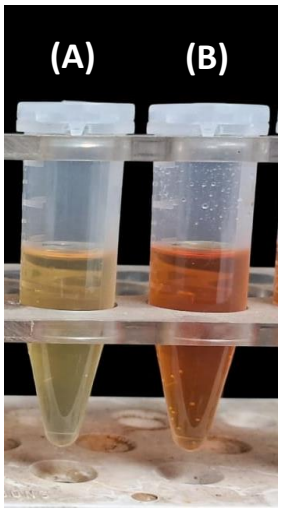


Figure 11. Detection of indole-3-acetic acid (IAA) production by bacterial isolates using Salkowski's reagent. (A) Control tube, (B) IAA-positive tube

1-Aminocyclopropane-1-carboxylic acid (ACC) deaminase activity, which helps mitigate ethylene-induced stress in plants, was evaluated on minimal Dworkin and Foster (DF) medium supplemented with 3 M of ACC. Growth indicates ACC utilization. A representative image has been included to illustrate the visual screening approach (Figure 12).

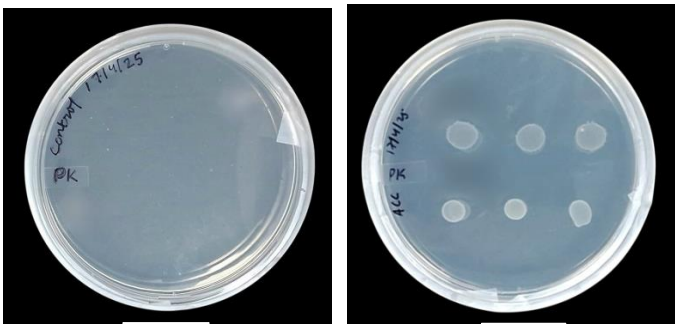


Figure 12. Screening of bacterial isolates for 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity on Dworkin and Foster (DF) minimal medium supplemented with 3 M ACC. (A) Control plate, (B) ACC-positive plate

Phosphate solubilization was tested on Pikovskaya's agar. Clear halo formation around the colonies indicates their ability to solubilize inorganic phosphate. A representative image has been included to illustrate the visual screening approach (Figure 13).

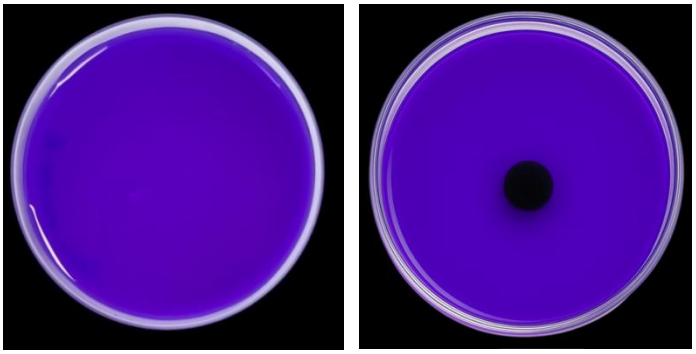


Figure 13. Screening of bacterial isolates for phosphate solubilization on Pikovskaya's agar plates. (A) Control plate, (B) phosphate solubilization-positive plate

Siderophore production was screened on Chrome Azurol S (CAS) agar. A yellow-orange halo, confirming siderophore production. A representative image has been included to illustrate the visual screening approach (Figure 14).

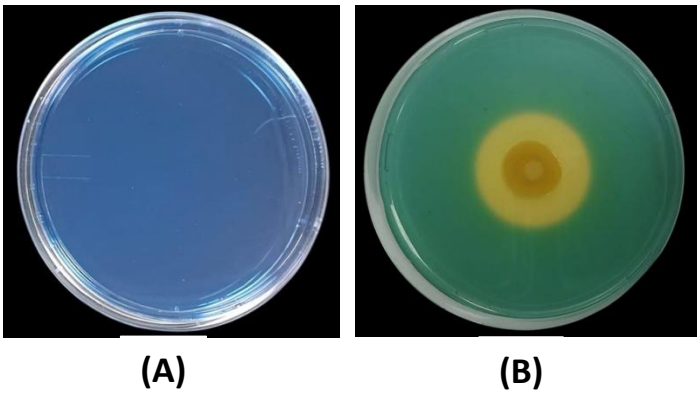


Figure 14. Detection of siderophore production in bacterial isolates on Chrome Azurol S (CAS) agar plates. (A) Control plate, (B) Siderophore-positive plate

Table 2. The table summarizes the strong activity (+), moderate or borderline activity (+/-), and weak or no activity (-) of key PGP traits: EPS production, IAA production, ACC deaminase activity, phosphate solubilization, and siderophore production in seven bacterial strains.

Bacterial strains	EPS	IAA	ACC	Phosphate solubilisation	Siderophore
PK1	+/-	+	+/-	+	+/-
PK3	+	+	-	+	+
PK5	+/-	+	+	+/-	+
PK6	+	+/-	+/-	+/-	+
PK7	+	-	-	+	+
PK8	+/-	+	+	+	-
PK10	+	+/-	+	+/-	-

Quantitative evaluation of plant growth-promoting (PGP) traits

The seven drought-tolerant bacterial isolates (PK1, PK3, PK5, PK6, PK7, PK8, and PK10) were subjected to quantitative assays to evaluate key plant growth-promoting (PGP) traits. These included precise measurements of their abilities to produce exopolysaccharide (EPS), indole-3-acetic acid (IAA), to express ACC deaminase activity, solubilize phosphate, and to produce siderophore.

The results for each trait are graphically illustrated below to highlight the comparative performance of the strains.

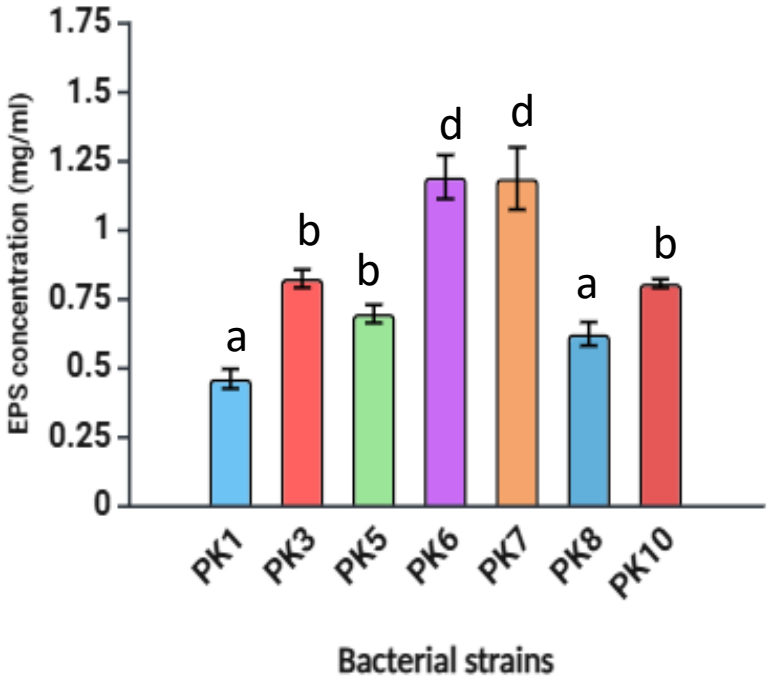


Figure 15. Quantitative estimation of EPS production in seven bacterial strains. Values represent mean ± SD of triplicates.

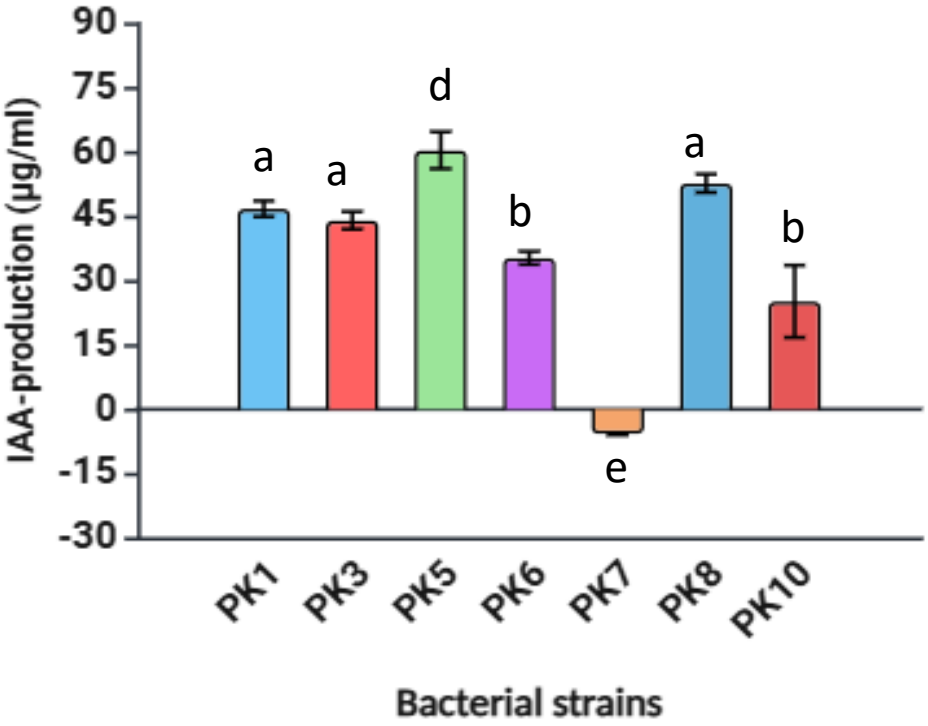


Figure 16. Quantitative estimation of IAA production in seven bacterial strains. Values represent mean ± SD of triplicates.

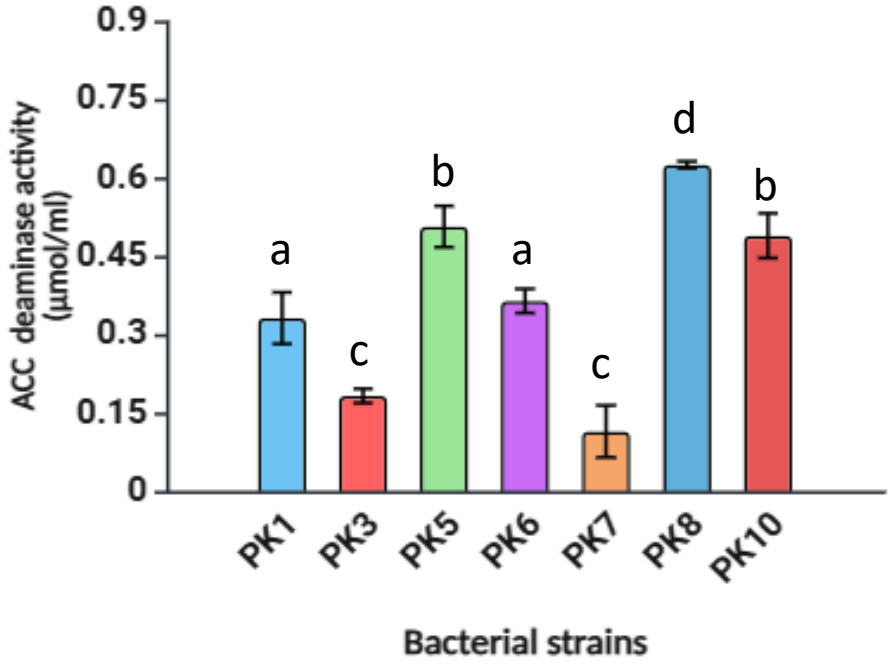


Figure 17. Quantitative estimation of ACC deaminase activity in seven bacterial strains. Values represent mean ± SD of triplicates.

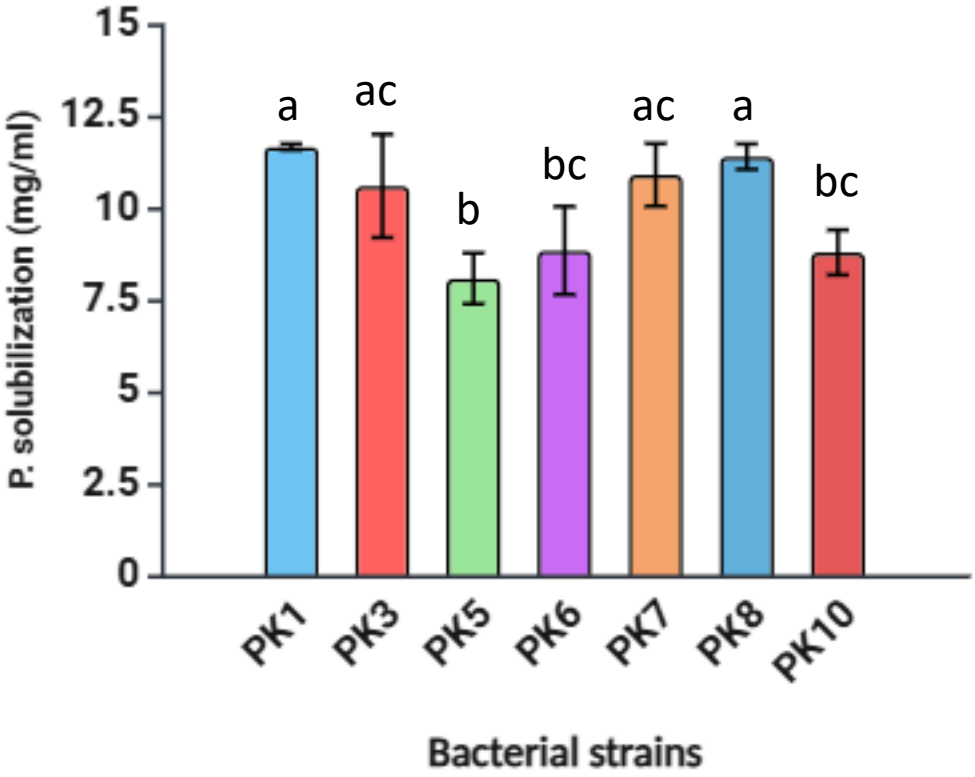


Figure 18. Quantitative estimation of phosphate solubilization in seven bacterial strains. Values represent mean ± SD of triplicates.

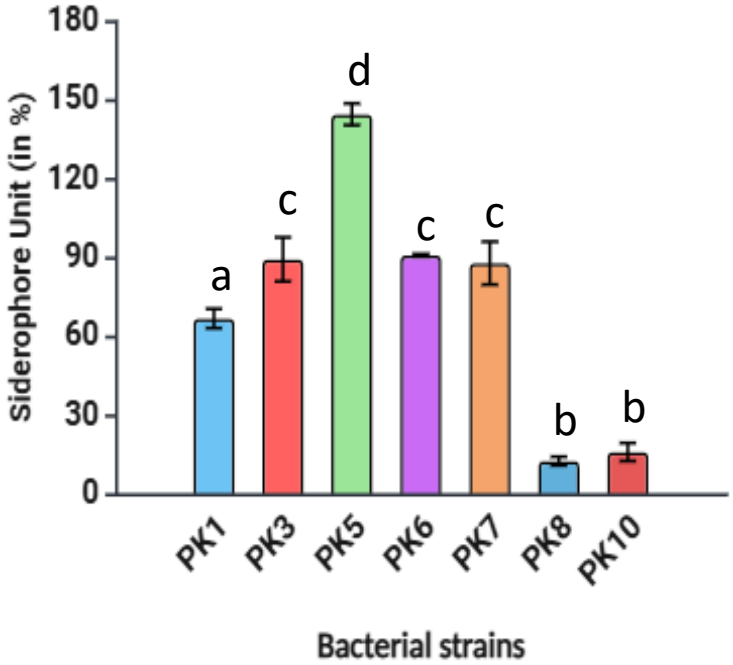


Figure 19. Quantitative estimation of siderophore production in seven bacterial strains. Values represent mean ± SD of triplicates.

Biofilm production and quantification

Biofilm formation ability of the seven bacterial strains (PK1, PK3, PK5, PK6, PK7, PK8, and PK10) was qualitatively and quantitatively assessed using crystal violet staining under normal (media + culture) and PEG-induced drought stress (media + PEG + culture) in microtiter plate. Visually, all strains exhibited some degree of biofilm formation at the air-liquid interface, with PK6 and PK10 exhibited significantly higher biofilm production in presence of PEG compared to their respective controls. Statistical analysis was done using paired t-test.

The results for each bacterial strain are graphically illustrated below (Figure 20).

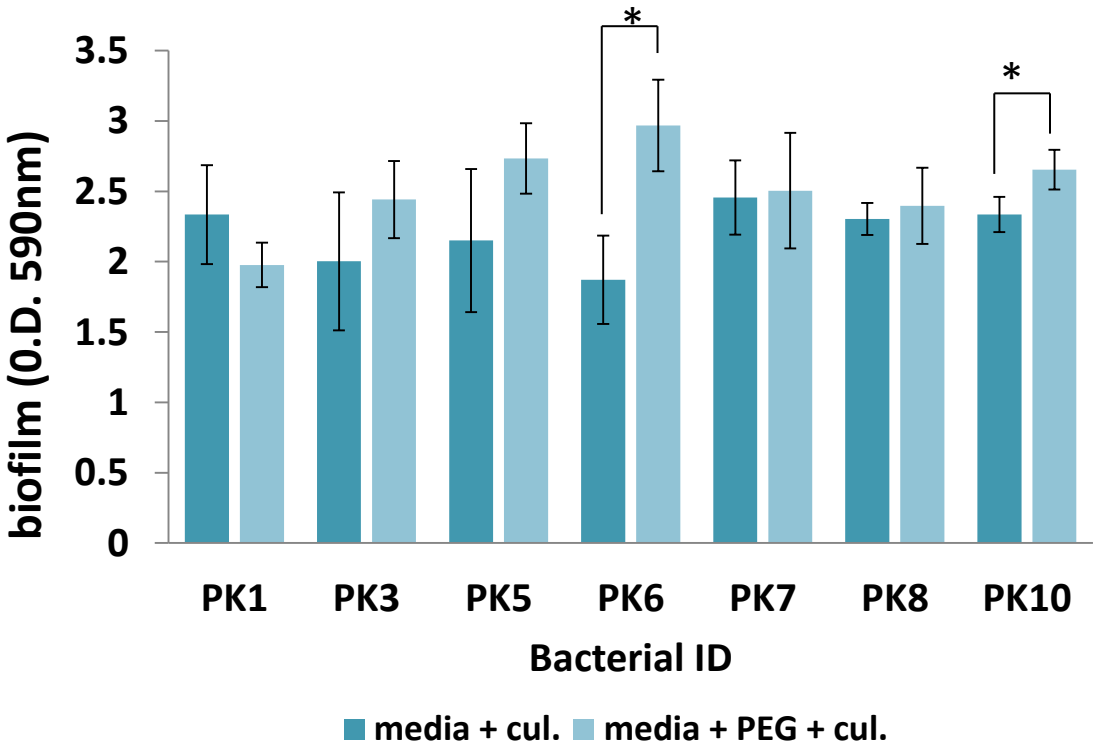


Figure 20. Quantitative estimation of biofilm formation in bacterial strains under controlled (media + culture) and PEG-induced (media + PEG + culture) osmotic stress conditions. Values represent mean ± SD of triplicates. * indicates statistical significance difference (*$p < 0.05$, **$p < 0.01$)

The compatibility of bacterial isolates was evaluated using the cross-streak assay on nutrient agar (NA) media, in which PK1 and PK3, PK5 and PK6, PK7 and PK10 are compatible to each other. However, PK8 was not compatible to any of the other bacterial strains.

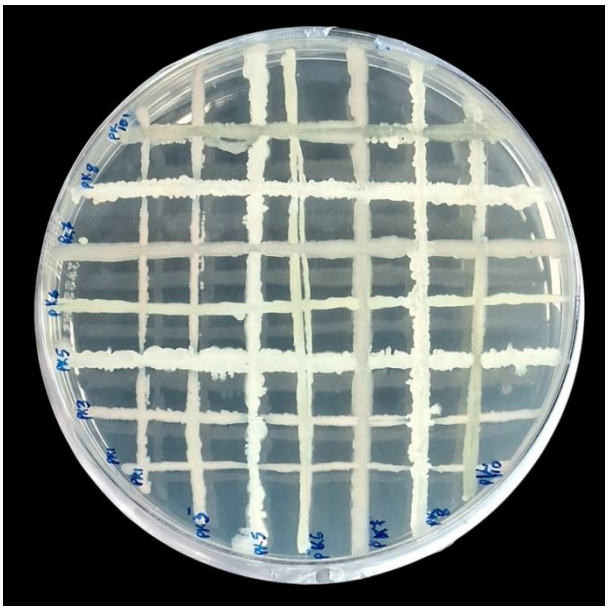


Figure 21. Compatibility testing of drought-tolerant bacterial strains via cross-streak assay.

Genomic DNA isolation and quantification

Genomic DNA was successfully extracted from all seven bacterial strains using CTAB (cetyltrimethylammonium bromide) extraction method. The quality and concentration of extracted DNA was evaluated using NanoDrop spectrophotometer. The DNA concentrations were found to range between 69.2 ng/µl and 1607.44 ng/µl. The A_{260}/A_{280} purity ratios ranged from 1.80 and 2.03, and A_{260}/A_{230} ratios ranged between 2.11 and 2.73, confirming acceptable purity for downstream molecular applications. The detailed DNA quantification and purity values are provided in Table 3.

Table 3. Quantification and quality check of isolated genomic DNA from bacterial strains.

Bacterial strains	Conc. (ng/µl)	260/280 ratio	260/230 ratio
PK1	1607.4	2.03	2.73
PK3	1468.3	2.01	2.73
PK5	349.1	1.90	2.58
PK6	69.2	1.80	2.44
PK7	355.7	1.97	2.70
PK8	114.3	1.82	2.11
PK10	1443.9	1.94	2.29

PCR amplification of 16S rRNA gene

After amplification the PCR products were analysed through agarose gel electrophoresis. A clear and distinct band was observed at 1500 bp for all seven bacterial strains, confirming successful amplification of the 16S rRNA gene. Notably, no band was observed in the negative control, validating the specificity and accuracy of the PCR setup (Figure 22).

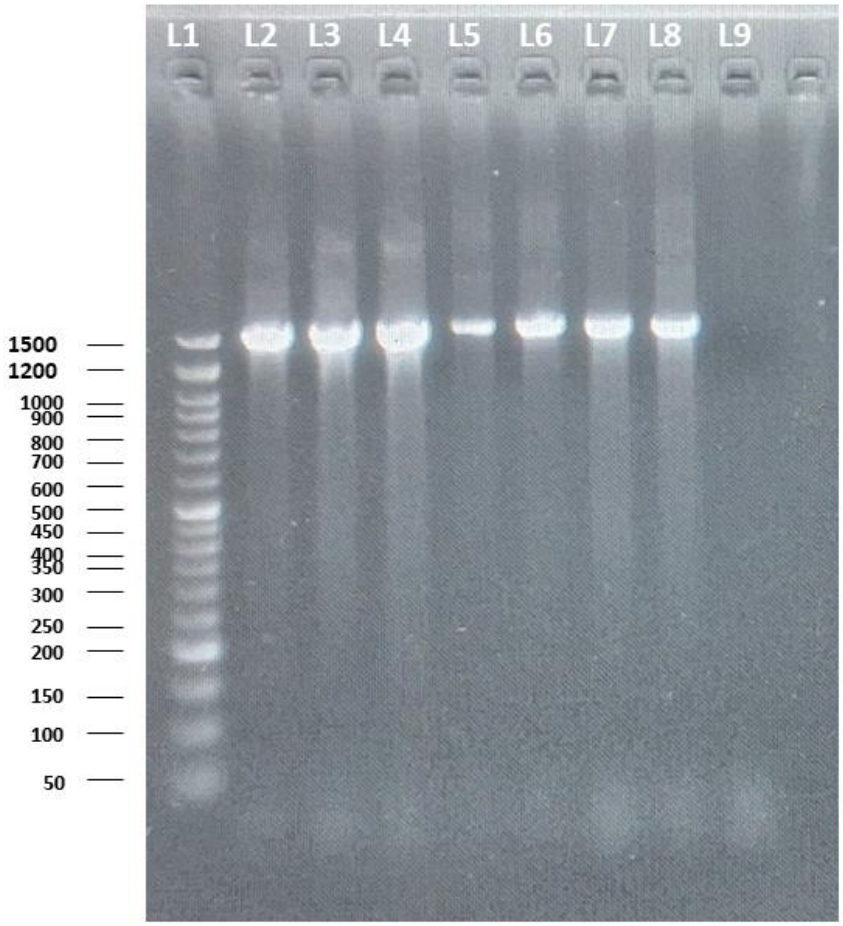


Figure 22. Agarose gel electrophoresis showing 16S rRNA gene amplification (~1500 bp) in seven bacterial strains with no amplification in the negative control.
Lane details: L1 – DNA ladder; L2 – PK1; L3 – PK3; L4 – PK5; L5 – PK6; L6 – PK7; L7 – PK8; L8 – PK10; L9 – negative control

Effect of Seed priming with EPS-producing bacterial isolateson tomato seedling growth under PEG-induced osmotic stress

Under *in vitro* conditions, tomato seedlings exhibited differential germination and growth responses across treatments. In the control condition (only water agar), 5 out of 9 seeds successfully germinated, demonstrating normal growth patterns. However, the PEG-induced osmotic stress treatment (water agar + 20% PEG-6000) resulted in a marked decline in germination, with only 3 out of 9 seeds showing emergence, indicating the inhibitory effect of osmotic stress on seed viability and early development. Notably, seeds primed with EPS-

positive strains prior to sowing on PEG-supplemented plate exhibited improved germination; with around 4-5 out of 9 seeds germinating per plate, which is closer to control and clearly improved over stress treatment. This suggests that EPS-primed conferred partial alleviation of osmotic stress, possibly through enhanced stress tolerance mechanism or improved seed vigor.

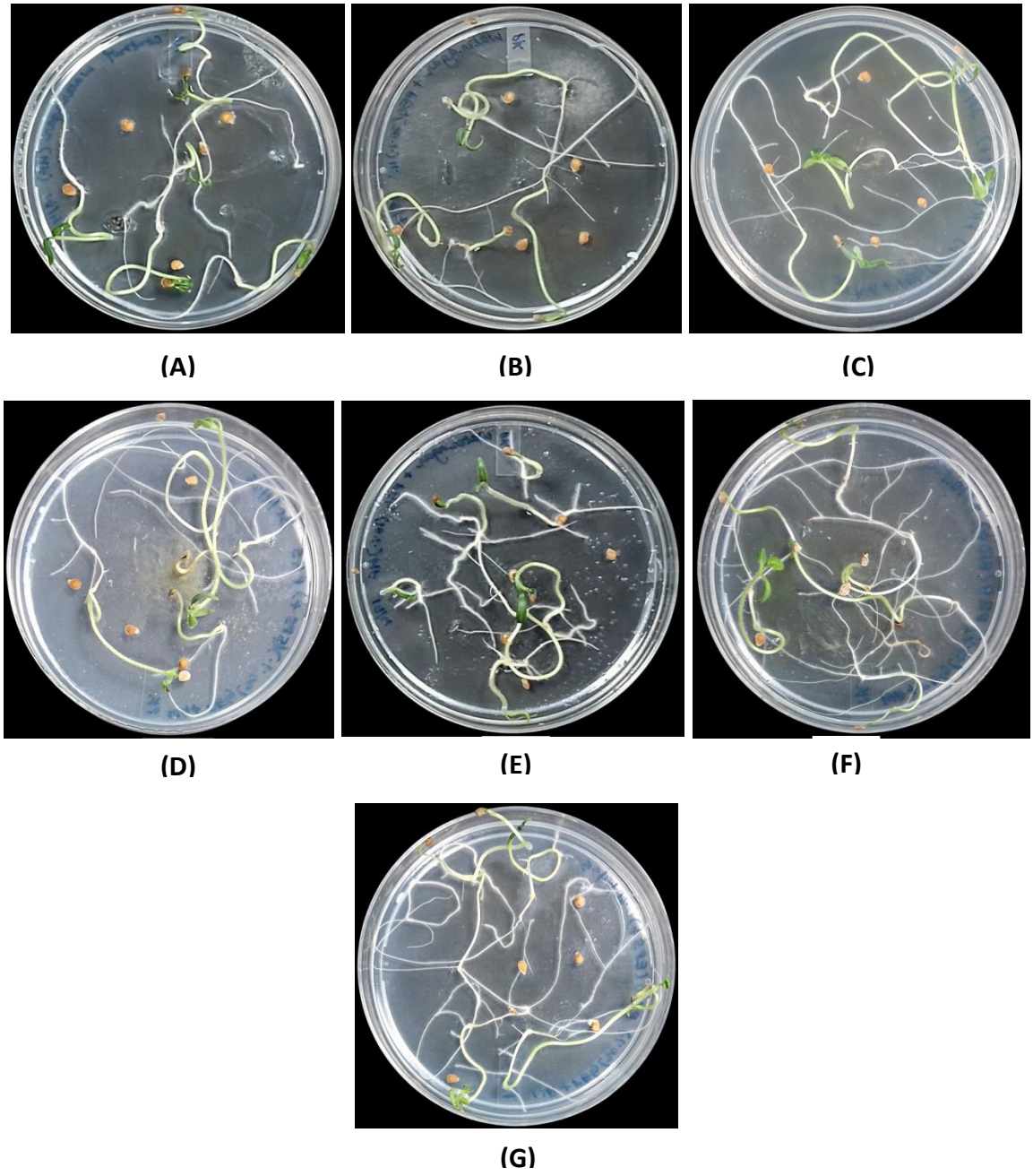


Figure 23. Germination and early seedling growth of tomato under controlled, PEG-induced osmotic stress, and PEG-induced osmotic stress + EPS-positive strain primed conditions. (A) Control (water agar) (B) PEG-induced osmotic stress (water agar + 20% PEG-6000), (C) seeds primed with EPS-positive PK1 strain, (D) seeds primed with EPS-positive PK3 strain, (E) seeds primed with EPS-positive PK5 strain, (F) seeds primed with EPS-positive PK8 strain, (G) seeds primed with EPS-positive PK10 strain.

6. Discussion

Rhizosphere engineering has emerged as an eco-friendly and sustainable approach to improve crop productivity and enhance stress tolerance through targeted manipulation of soil microbiota. In alignment with this concept, the current study explores a novel approach involving the transplantation of an acclimatized rhizospheric microbiome to tomato plants. This method, rooted in a top-down rhizosphere engineering strategy, holds promise for promoting drought resilience while supporting sustainable agricultural practices. Notably, only limited research has applied such microbiome-based interventions in tomato, underscoring the innovative nature of the present investigation.

The present study aimed to evaluate the potential of native rhizospheric bacteria to enhance tomato growth under drought conditions through a stepwise experimental framework. The initial phase of plant acclimatization across three successive potting cycles helped establish a stable experimental system and minimized variability across treatments. This phase was crucial in refining the stress induction protocol, particularly with respect to drought exposure timing and inoculum application, ensuring that subsequent analyses reflected genuine physiological plant responses.

Isolation of bacterial strains from the rhizosphere during the first cycle revealed a diverse microbial population, from which a subset was selected based on their consistent growth in tomato root exudate (TRE) mimicking medium and polyethylene glycol (PEG)-simulated drought conditions. The screening of these isolates for plant growth-promoting (PGP) traits revealed variability in functional attributes. Notably, the presence of traits such as indole-3-acetic acid (IAA) production, ACC deaminase activity, phosphate solubilization, and siderophore production in several isolates suggests their potential role in modulating plant hormonal balance, enhancing nutrient uptake, and alleviating abiotic stress responses. These functional traits align with previous studies, reinforcing the significance of such microbial traits in conferring drought resilience.

Overall, this study underscores the potential of microbiome-assisted strategies as a viable tool for enhancing drought tolerance in crops, paving the way for more resilient and sustainable agricultural systems.

7. Conclusion

This study represents a novel integration of biological and physical methodologies to evaluate the impact of microbial inoculants on drought stress mitigation in tomato. A multi-passaging experimental framework was employed to systematically investigate the role of native plant-growth-promoting rhizobacteria (PGPR) in enhancing drought tolerance.

The first objective focused on conducting a three-cycle multi-passaging experiment to acclimatize tomato seedlings under drought conditions. By the third cycle, consistent and significant improvements were observed in drought stress indicators—including proline accumulation, malondialdehyde (MDA) levels, and total chlorophyll content—particularly in the stress + inoculum (S+I) treatments compared to the stress-only (S) group, across both autoclaved and unautoclaved inoculum sets.

The second objective involved isolating PGPR strains from the rhizospheric soil of the initial plant growth cycle. These isolates were subjected to qualitative and quantitative screening for key PGP traits such as exopolysaccharide (EPS) production, indole-3-acetic acid (IAA) synthesis, ACC deaminase activity, phosphate solubilization, and siderophore production. Genomic DNA was extracted from the selected strains, followed by successful 16S rRNA gene amplification, with distinct bands at ~1500 bp confirming PCR specificity.

To fulfil the final objective of my study, EPS-mediated priming was evaluated for its impact on tomato seed germination under PEG-induced osmotic stress. EPS priming of tomato seeds with selected bacterial isolates resulted in improved germination under PEG-induced osmotic stress, compared to stress-only treatment. These findings indicate that EPS-producing bacteria may contribute to early-stage drought resilience in tomato seedlings.

8. Future prospects

The multi-passaging experiment will be extended up to at least 10 plant growth cycles to achieve full acclimatization of tomato seedlings under drought stress. Bacterial strains will be isolated from each cycle from the rhizospheric soil to monitor changes in the rhizosphere microbiome and identify novel, stress-adapted PGPR strains. These strains will be screened for plant growth-promoting (PGP) traits to expand the pool of effective candidates. Additionally, the bacterial strains previously isolated from first plant growth cycle subjected to 16S rRNA amplification will undergo sequencing and taxonomic identification.

In the long-term, promising bacterial strains will be assembled into synthetic microbial communities (SynComs) and tested for field trial, with the goal of developing effective bioinoculant formulations for sustainable crop improvement under drought conditions.

Appendix

Composition of M9 minimal supplemented with tomato root exudate (TRE) components

1. Basal M9 medium composition

Components	**Concentration**
Disodium hydrogen phosphate (Na_2HPO_4)	6.78 g/l
Potassium dihydrogen phosphate (KH_2PO_4)	3 g/l
Sodium chloride (NaCl)	0.5 g/l
Ammonium chloride (NH_4Cl)	0.5 g/l
Potassium nitrate (KNO_3)	0.5 g/l
Magnesium sulphate ($MgSO_4$, 1M)	2 ml/l
Calcium chloride ($CaCl_2$, 1M)	100/l

2. Carbon source (autoclaved separately and added post-sterilization)

Components	**Concentration**
Glucose ($C_6H_{12}O_6$)	0.5% (w/v)
Fructose ($C_6H_{12}O_6$)	0.5% (w/v)

3. Organic acids (added as 50X stock)

Components	**Concentration**
Citric acid ($C_6H_8O_7$)	110 mg/l
Malic acid ($C_4H_6O_5$)	2.3 mg/l
L-Glutamic acid ($C_5H_9NO_4$)	61.5 mg/l

4. Trace elements (added as 50X stock)

Element	**Components**	**Concentration**
Iron (Fe^{3+})	Ferric chloride hexahydrate ($FeCl_3.6H_2O$)	1 µg/l
Copper (Cu^{3+})	Copper(II) sulafate pentahydrate ($CuSO_4.5H_2O$)	78.12 ng/l

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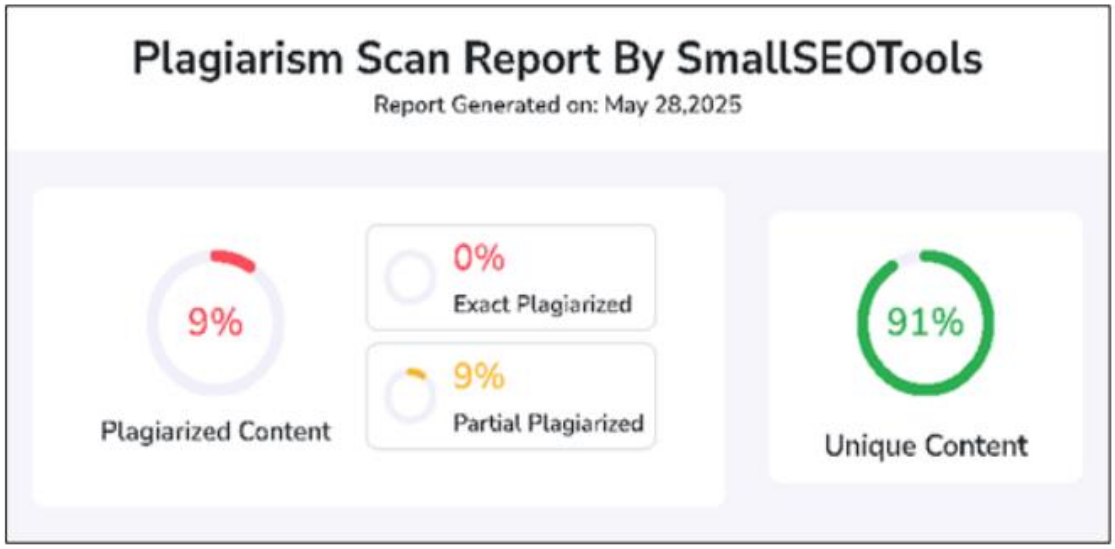
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